STUDIES WITH TISSUE CULTURES OF TRIPTERYGIUM WILFORDII.
ISOLATION OF METABOLITES AND BIOTRANSFORMATION STUDIES

By
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We accept this thesis as conforming
to the required standard

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Department of **CHEMISTRY**

The University of British Columbia
Vancouver, Canada

Date 18/1/90
ABSTRACT

In a program aimed at the identification of compounds responsible for the immunosuppressive and antifertility activities of the perennial twining vine, *Tripterygium wilfordii*, 5 new and 13 known compounds were isolated from the TRP-4a tissue culture cell line developed from *Tripterygium wilfordii*. The structures of the new compounds were determined by a combination of spectral analysis, chemical correlation and single crystal X-ray diffraction analysis.

22β-Hydroxy-3-oxoolean-12-en-29-oic acid (137), 22α-hydroxy-3-oxoolean-12-en-29-oic acid (138) and 3β, 22β-dihydroxyolean-12-en-29-oic acid (139) are new triterpenes possessing an oleanene-type skeleton and were chemically correlated with 3β, 22α-dihydroxyolean-12-en-29-oic acid (51), the structure of which was confirmed by single crystal X-ray diffraction analysis. Oleanolic acid (127), β-sitosterol (128) and polpunonic acid (55), were isolated previously from the TRP-4a cell line in earlier studies in this laboratory.

α-Amyrin (145), β-amyrin (146), 3β, 29-dihydroxyolean-12-ene (151) and 3β, 11α-dihydroxyolean-12-ene (152) are known triterpenes possessing an oleanene-type skeleton and are isolated for the first time from the TRP-4a cell line. Tingenone (148) and 22β-hydroxytingenone (150) are quinone methide triterpenes, also isolated for the first time from the TRP-4a cell line. Similarly, the novel diterpene, 12-methoxyabieta-8, 11, 13-trien-3α-ol (147) and the novel triterpene, methyl-22β-hydroxy-3, 21-dioxo-D:A-friedo-29-noroleanan-24-oate (149), a member of the friedelane family, are isolated for the first time. A biosynthetic pathway, based on the isolation of 149 and its structural similarity to polpunonic acid (55) and 22β-hydroxytingenone (150), is postulated for the quinone methides.

The cytotoxic diterpenes, tripdiolide (1) and triptolide (2) and the hydroxy acid, 160, isolated as the methyl ester, 124, have been previously reported from this laboratory. Tripdiolide (1) and triptolide (2) have been shown to possess strong antifertility and immunosuppressive activities.
In another aspect of our program, biotransformation studies of the synthetic precursors, 19 (4→3)abeo-abieta-2, 8, 11, 13-tetraen-19-ol (171) and 19-hydroxy-18(4→3)abeo-abieta-3, 8, 11, 13-tetraen-18-oic acid lactone (91), and the radioactive congeners, 182 and 209, were carried out using the TRP-4a cell line. It was hoped that the data obtained might shed some light on the “late stage” biosynthetic pathway of the diterpene triepoxides, triptolide (1) and triptolide (2).

Synthesis of 171 was achieved in 5 steps from dehydroabietic acid (80). The radioactive congener, 182, was synthesised using $^{14}$C-paraformaldehyde with 0.4% incorporation of the radiolabel. Biotransformation of 171 using the TRP-4a cell line yielded 19(4→3)abeo-abieta-2, 8, 11, 13-tetraen-19-al (185) and 19(4→3)abeo-abieta-2, 8, 11, 13-tetraen-19-oic acid (186) for spectral identification. Biotransformation of 182 yielded the aldehyde, 183 (33.2%) and the acid, 184 (51.9%), the radioactive congeners of 185 and 186 respectively.

Synthesis of 91 was achieved in 4 steps from dehydroabietic acid (80). The radioactive congener, 209, was synthesised using $^{14}$C-methyl iodide via $^{14}$C-dimethylsulphonium methyldide, with 0.6% incorporation of the radiolabel. Biotransformation of 91 using TRP-4a tissue cultures yielded 19-hydroxy-7-oxo-18(4→3)abeo-abieta-3, 8, 11, 13-tetraen-18-oic acid lactone (214), 2β, 19-dihydroxy-7-oxo-18(4→3)abeo-abieta-3, 8, 11, 13-tetraen-18-oic acid lactone (215), 7β, 19-dihydroxy-18(4→3)abeo-abieta-3, 8, 11, 13-tetraen-18-oic acid lactone (216) and 2β, 19-dihydroxy-18(4→3)abeo-abieta-3, 8, 11, 13-tetraen-18-oic acid lactone (96), for spectral identification. Biotransformation of 209 yielded the ketone, 210 (56.7%), the hydroxy ketone, 211 (5.9%), the benzylic alcohol, 212 (9.6%) and the C2 alcohol, 213 (6.8%), the radioactive congeners of 214, 215, 216 and 96 respectively. A biosynthetic pathway to the diterpene triepoxides is postulated based on the oxygenated biotransformation products.
The numbering system used throughout this work is that used by contemporary natural products chemists and is illustrated below. The lettering of the rings is given in alphabetical order and proceeds from left to right across the structure.
The abietane skeleton is designated as 18-nor if C18 is absent or if a double bond is present at C3 or C4, and as 19-nor if the remaining methyl group at C4 is α. The rearranged abietane skeleton is designated as 18(4→3)abeo except if the C4 methyl group is α, then the skeleton is designated as 19(4→3)abeo.
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<tr>
<td>APT</td>
<td>Attached proton test</td>
</tr>
<tr>
<td>B-5</td>
<td>Standard tissue culture medium developed by Gamborg and Eveleigh&lt;sup&gt;50&lt;/sup&gt;</td>
</tr>
<tr>
<td>br</td>
<td>Broad</td>
</tr>
<tr>
<td>COSY</td>
<td>2-Dimensional correlated NMR spectroscopy</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>d</td>
<td>Doublet</td>
</tr>
<tr>
<td>dd</td>
<td>Doublet of doublets</td>
</tr>
<tr>
<td>ddd</td>
<td>Doublet of doublet of doublets</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPM</td>
<td>Disintegrations per minute</td>
</tr>
<tr>
<td>dt</td>
<td>Doublet of triplets</td>
</tr>
<tr>
<td>ED&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Median effective dose</td>
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<td>GTW</td>
<td>A multi-glycoside extract from the plant <em>Tripterygium wilfordii</em></td>
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<td>HC50</td>
<td>50% Haemolytic concentration</td>
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<td>HPLC</td>
<td>High pressure liquid chromatography</td>
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<td>HRMS</td>
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<td>Generally a mixture of several isomers of hexane (C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;14&lt;/sub&gt;), predominantly n-hexane, and methylcyclopentane (C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;) (BDH)</td>
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<td>J</td>
<td>Coupling constant</td>
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<td><strong>LD₅₀</strong></td>
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<td><strong>m</strong></td>
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<td>Nuclear Overhauser effect</td>
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<td><strong>PCC</strong></td>
<td>Pyridinium chlorochromate</td>
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<tr>
<td><strong>PRD₂Co₁₀₀</strong></td>
<td>PRL-4 medium of Gamborg and Eveleigh superscript 50 supplemented with 2,4-dichlorophenoxyacetic acid (D, 2 mg/L) and coconut milk (Co, 100 mL/L)</td>
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<tr>
<td><strong>PRI₂Co₁₀₀</strong></td>
<td>PRL-4 medium of Gamborg and Eveleigh superscript 50 supplemented with indole-3-acetic acid (I, 2 mg/L) and coconut milk (Co, 100 mL/L)</td>
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<td><strong>PRL-4</strong></td>
<td>Standard tissue culture medium developed by Gamborg and Eveleigh superscript 50</td>
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<tr>
<td><strong>PVP</strong></td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td><strong>RDA</strong></td>
<td>Retro Diels-Alder fragmentation</td>
</tr>
<tr>
<td><strong>RE</strong></td>
<td>Rabbit erythrocytes (rosette formation)</td>
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<td><strong>s</strong></td>
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<td><strong>sh</strong></td>
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<tr>
<td><strong>SINEPT</strong></td>
<td>Selective insensitive nucleus enhancement by polarization transfer</td>
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<tr>
<td><strong>t</strong></td>
<td>Triplet</td>
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<tr>
<td><strong>THF</strong></td>
<td>Tetrahydrofuran</td>
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<tr>
<td><strong>TMEDA</strong></td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td><strong>TMS</strong></td>
<td>Tetramethylsilane</td>
</tr>
<tr>
<td><strong>Tosyl (Ts)</strong></td>
<td>para-Toluenesulphonyl (the abbreviation tosyl is employed in the text while Ts is employed in structures)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>Tosylate</td>
<td><em>para</em>-Toluenesulphonate</td>
</tr>
<tr>
<td>Triflate</td>
<td>Trifluoromethanesulphonate</td>
</tr>
<tr>
<td>Trisyl</td>
<td>2, 4, 6-Triisopropylbenzenesulphonyl</td>
</tr>
<tr>
<td>TRP-4a</td>
<td>A cell line of tissue culture developed from <em>Tripterygium wilfordii</em></td>
</tr>
<tr>
<td>VLC</td>
<td>Vacuum liquid chromatography</td>
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<td>w</td>
<td>Weak</td>
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I would like to thank my supervisor, Professor J.P. Kutney for his guidance and support throughout this work. I would like to thank also the following people for their contribution:

Dr. Krystyna Piotrowska, Mijo Samija, Francisco Kuri-Brena and Phil Gunning for their help and invaluable suggestions during the preparation of this thesis;

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1 INTRODUCTION

1.1 Background

*Tripterygium wilfordii* Hook f is a perennial twining vine of the family Celastraceae, which is cultivated in many parts of southern China such as Zhejiang, Anhui, Jiangxi, Fujian and Guangdong provinces and also in Taiwan. The herb is commonly known in China as Lei Gong Teng (Thunder God vine) or Mang Cao (rank grass). Its use in Chinese traditional medicine dates back many centuries, and it is first mentioned in the *Saint Peasant's Scripture of Materia Medica*, written about two thousand years ago, as being used for the treatment of fever, chills, oedema and carbuncle. Chinese gardeners used the powdered root to protect their crops from chewing insects. Most recently, crude extracts and refined extracts (a so-called multi-glycoside extract, or GTW) have been used increasingly to treat such disorders as rheumatoid arthritis, ankylosing spondilitis and a variety of dermatological disorders.\(^2\)\(^3\)\(^4\)

![Chemical structures](image-url)
In 1972, Morris Kupchan and co-workers first isolated the novel diterpenoid triepoxides, triptolide (1), triptolide (2), and triptonide (3) from the roots of *Tripterygium wilfordii*. These were the first reported natural products containing the 18(4→3) abeo-abietane skeleton and the first recognised diterpenoid triepoxides. Triptolide (1) and triptolide (2) were shown to have significant antileukaemic activity and these data generated a great deal of interest in these compounds. Since then, much research has been carried out into the production of the diterpene triepoxides for further biological evaluation. Numerous compounds have been isolated from *Tripterygium wilfordii* plants and their biological properties investigated (*vide infra*).

1.2 The chemistry of *Tripterygium wilfordii*

1.2.1 Alkaloids

Acree and Haller first isolated the insecticidal sesquiterpene alkaloid mixture, designated "wilfordine," from the roots of *Tripterygium wilfordii*. Beroza, in 1953, showed that this mixture consisted of five alkaloids, wilforine (4), wilforgine (5), wilfordine (6), wilfortrine (7), and wilforzine (8) with molecular formulae $\text{C}_{43}\text{H}_{49}\text{NO}_{18}$, $\text{C}_{41}\text{H}_{47}\text{NO}_{19}$, $\text{C}_{43}\text{H}_{49}\text{NO}_{19}$, $\text{C}_{41}\text{H}_{47}\text{NO}_{20}$, and $\text{C}_{41}\text{H}_{47}\text{NO}_{17}$ respectively. Degradation studies revealed that all 5 alkaloids contained a polyhydroxy nucleus with the basic formula $\text{C}_{15}\text{H}_{26}\text{O}_{10}$; alkaloids 4 to 7 contained 5 acetyl units, while alkaloid 8 contained only 4; alkaloids 4, 6 and 8 contained a benzoate ester while 5 and 7 contained a furoate ester; and that alkaloids 4, 5 and 8 contained a dibasic acid of molecular formula $\text{C}_{11}\text{H}_{13}\text{NO}_{4}$, while alkaloids 6 and 7 contained a hydroxy dibasic acid of molecular formula $\text{C}_{11}\text{H}_{13}\text{NO}_{5}$. Shizuri et al. identified wilfordine (6) isolated from *Euonymus alatus* forma *striatus* (thunb.) Makino, by spectroscopic and chemical methods and by chemical correlations. Wilforine (4), wilfordine (6) and wilfortrine (7) were later identified by chemical and spectroscopic methods together with a new alkaloid wilforidine (9).
The structures of wilforgine (5), wilforzine (8) and a further new alkaloid, wilformine (10) were determined shortly after the above studies. Wilfortrine (7) was shown to inhibit leukaemia cell growth in mice. Deng et al. isolated and identified euonine (identical to wilformine (10)) and wilfornine (11) by spectroscopic methods and showed them to have immunosuppressive activities in mice.

Takaishi et al. isolated five new alkaloids from the leaves of *Tripterygium wilfordii* Hook fil. var. *regelii* Makino, a related species, and determined the structures of two of them, triptofordinine A-1 (12) and A-2 (13) by 2D-NMR spectroscopy including $^1$H-$^1$C long range COSY experiments. Hori et al. isolated the alkaloid regilidine (14) from the roots of the same plant, and determined its structure by spectroscopic methods. The absolute stereochemistry was established by X-ray crystallography.

Kupchan et al. isolated the novel macrocyclic spermidine alkaloids celacinnine (15), celabenzine (16) and celafurine (17) from the roots of *Tripterygium wilfordii*. Their structures were established spectroscopically and by chemical methods.
1.2.2 Sesquiterpenes

Takaishi et al. have isolated several sesquiterpene esters: triptofordins A, B, C-1, C-2, D-1, D-2, E (18 to 24 respectively) and two previously known sesquiterpene esters, 25 and 26. More recently, the same group identified four more sesquiterpene esters: triptofordins F-1 to F-4 (27 to 30 respectively). All of the compounds were isolated from the leaves of the related species *Tripterygium wilfordii* Hook fil. var. *regelii* Makino. The above sesquiterpene esters were shown to possess the same basic agarofuran skeleton (shown as structure 31) which also constituted the sesquiterpene moiety of the aforementioned sesquiterpene alkaloids.
18 $R^1=H$, $R^2=\text{COCH}=\text{CHPh}$
19 $R^1=\text{OH}$, $R^2=\text{COPh}$
20 $R^1=R^3=\text{Ac}$, $R^2=\text{O}$
21 $R^1=R^3=\text{Ac}$, $R^2=\text{H}$, $\beta-\text{OH}$
22 $R^1=\text{Ac}$, $R^2=\text{O}$
23 $R^1=\text{Ac}$, $R^2=\text{H}$, $\beta-\text{OH}$
24 $R=\text{COPh}$
25 $R=\text{Ac}$
26 $R=\text{COCH}=\text{CHPh}$
27 $R^1=H$, $R^2=\text{Ac}$, $R^3=\text{COPh}$, $R^4=\text{COCH}=\text{CHPh}$
28 $R^1=H$, $R^2=R^3=\text{COPh}$, $R^4=\text{Ac}$
29 $R^1=R^2=\text{Ac}$, $R^3=R^4=\text{COPh}$
30 $R^1=R^2=\text{H}$, $R^3=\text{COPh}$, $R^4=\text{COCH}=\text{CHPh}$
Two norsesquiterpenes have been isolated from *Tripterygium wilfordii* and their structures have been determined. Wilforonide (32)\(^{21}\) and neotriptonolide (33)\(^{22}\) both contain the \(\alpha\beta\)-unsaturated \(\gamma\)-lactone in ring A, characteristic of the diterpene triepoxides.

1.2.3 Diterpenes

Aside from the aforementioned diterpene triepoxides, tripdiolide (1), triptolide (2) and triptonide (3),\(^5\) various other abietane-type diterpenes have been isolated from *Tripterygium wilfordii*. Triptonolide (34),\(^{23}\) triptophenolide (35), triptophenolide methyl ether (36), neotriptophenolide (37)\(^{24}\) and isoneotriptophenolide (38)\(^{21}\) all exhibit the rearranged 18(4→3) *abeo*-abietane skeleton with varying degrees of oxygenation at the B/C ring system. A new diterpene triepoxide, triptolidenol (39), recently isolated,\(^{25}\) possesses a hydroxyl group at C15.

Other abietane-type diterpenes have been isolated which do not possess the lactone ring structure in ring A but show oxygen functionality in ring C. Zhu et al. isolated the diterpenes triptonoterpene (40) and triptonoterpene methyl ether (41),\(^{25}\) while Zhou et al. isolated the isomeric neotriptonoterpene (42) and triptonodiol (43), possessing an \(\alpha\)-hydroxymethyl group at C3.\(^{22}\) The diterpene quinones, 44 and 45, have been isolated from the related species *Tripterygium regelii*.\(^{26}\)
1.2.4 Triterpenes

Since the isolation of celastrol (tripterine (46))\textsuperscript{27,28} in 1936, several triterpenes of the oleanene, friedelane and ursene type have been isolated from \textit{Tripterygium wilfordii}. Qin et al. isolated the triterpene lactones wilforlides A and B (47 and 48 respectively) from the roots of the plant and identified them by spectroscopic and chemical methods.\textsuperscript{29} The novel dihydroxy triterpene acid, triptotriterpenic acid A (49)\textsuperscript{30} and its lactone congener, triptotriterpenoidal lactone A (50),\textsuperscript{31} were also isolated from the roots of the plant. Triptotriterpenic acid A (49) was found to be effective in antiinflammation. The same research group isolated 3\textbeta,22\alpha-dihydroxyolean-12-en-29-oic acid (51) from the roots which also exhibited antiinflammatory activity in laboratory animals.\textsuperscript{32}
Zhang et al. isolated the oleanene 3-epikatonic acid (52) and two friedelane-type compounds, salaspermic acid (53) and the novel compound, tentatively assigned as 3,24-dioxofriedelan-29-oic acid (54). Also isolated were the wilforlides A and B (47 and 48), the dihydroxy compound, 51 and celastrol (46). Celastrol was found to inhibit proliferation of lymph cells. Polpunonic acid (55) and a new triterpene, triptodihydroxy acid methyl ester (56) have also been isolated.

The ursene-type triterpenoids, regelin (57) and regelinol (58) were isolated from the related species Tripterygium regelii together with wilforlide A (47). The structures of regelin and wilforlide A were confirmed by X-ray analysis.
1.3 Biological activities and structure-activity relationships of the diterpene triepoxides

The interesting biological activities exhibited by the diterpene triepoxides, isolated by Kupchan, led to investigations into their structure-activity relationships. Biological evaluations of these compounds showed that tripdiolide (1) and triptolide (2) exhibited significant activity in vivo against the L-1210 and P-388 leukaemias in the mouse and in vitro against cells derived from human carcinoma of the nasopharynx (KB).\textsuperscript{5,35,36} Impressive life-prolonging effects were demonstrated at the 0.1 mg/kg level for both compounds when tested against the L-1210 lymphoid leukaemias, and cytotoxicity
(ED$_{50}$) against KB cells was demonstrated at $10^{-3}$ to $10^{-4}$ µg/mL. Triptonide (3) however, showed no antileukaemic activity in doses up to 0.4 mg/kg.

These results led to the proposal by Kupchan that the 14β-hydroxyl and 9,11-epoxide groups were necessary for the antileukaemic activity of the diterpene triepoxides, and that the 14β-hydroxyl group participated in the opening of the epoxide group during alkylation of thiol groups (Figure 1). This hypothesis is based on the proposal that other plant-derived tumour inhibitors may act via selective alkylation of the thiol groups of growth regulation enzymes. The fact that triptonide (3) and 14-epitriptolide (59) showed no significant activity against leukaemia supports the proposal that intramolecular catalysis by the 14β-hydroxyl group is taking place.

Figure 1 Alkylation of thiols by the diterpene triepoxides via hydroxyl-assisted epoxide ring opening

Analogues (61 to 65) of the above triepoxides were synthesised by Tokoroyama et al. (vide infra) and screened for antitumour activity against KB cells and L-1210
leukaemia in mice. The results indicated that not only the hydroxy-epoxide system in the B/C ring system, but also some functionality (possibly the butenolide ring) in ring A is necessary for the biological activities. Compound 66, synthesised by Berchtold et al., showed no antitumour activity, which further supports this conclusion.

More recent studies have shown that triptolide (2) prolonged the survival time of L615-bearing mice, with some mice surviving for longer than a month. These mice were rechallenged with leukaemic cells with no recurrence of the disease. Toxicity studies of triptolide (2) have shown that the LD$_{50}$ for mice was 0.8 mg/kg after intravenous administration. Dogs given triptolide intravenously, at 20-160 μg/kg/day, showed pathological or functional changes in the heart, liver, and gastrointestinal tract.
1.4 Methods for the production of the diterpene triepoxides

The very promising antineoplastic activities of the diterpene triepoxides has stimulated research into their production in greater quantities. Any future clinical trials can only continue when sufficient amounts of the products become available. Their commercial value as antineoplastic agents is also potentially quite high. Three methods available for the production of natural products include:

1) isolation from the intact plant;
2) total synthesis;
3) isolation from tissue cultures of the plant.

For large scale production of medicinal agents, however, various problems are inherent with isolation from higher plants. Usually, such sources result in isolation of only minute quantities of the desired compound, and because of the complexity of the crude extracts, separation from co-occurring materials is often difficult, costly and time-consuming. Furthermore, the concentration of the desired compound may vary according to the time of harvesting of the plant. Other constraints are incurred by the fact that the plants may grow very slowly, and environmental and geopolitical constraints may make the required plant species unavailable.

Research into the total synthesis of triptonide (3) and triptolide (2) has been carried out by a number of research groups. The total syntheses to date, however, suffer from the disadvantages that many steps are involved, and overall yields are quite low, rendering them unsuitable for commercial-scale production. Tripdiolide (1) has, to date, not been synthesised.

The problems associated with these first two methods has stimulated research into the development of plant tissue cultures for the production of the diterpene
triepoxides. The advantages of tissue culture techniques lie in the facts that growth conditions can be controlled and are, therefore, reproducible; growth parameters can be manipulated in order to optimize production of the target compound; and selected cell lines can be provided by means of cloning for reliable production of the target compound. Tissue cultures also provide excellent media for biosynthetic studies, and enzyme isolations are potentially more feasible to provide a better understanding of the biosynthetic processes.

1.4.1 Isolation of the diterpene triepoxides from the intact plant

Kupchan's first isolation of the diterpene triepoxides was carried out by extraction from 2 kg of ground roots and, following a series of chromatographic separations, resulted in the isolation of 26 mg of tripdiolide (1), 21 mg of triptolide (2) and 20 mg of triptonide (3). This illustrates the low yields (0.001%) available from plant sources. Research groups in China have since isolated tripdiolide from *Tripterygium wilfordii* and other species of *Tripterygium* such as *Tripterygium hypoglauca*.\(^4\) The observed low yields of these compounds obtainable from the intact plant are prohibitive for large scale production and therefore alternative sources must be sought.

1.4.2 Total syntheses of the diterpene triepoxides

A number of strategies have been adopted in the total synthesis of triptolide and triptonide. The major obstacles which had to be overcome were the construction of the triepoxide system in ring C and the butenolide ring in ring A. Approaches to these problems involved synthesis of the abietane skeleton incorporating functionality at the appropriate positions for further elaboration, and suitable derivatization of a readily available abietane-type starting material.

The approach to the synthesis adopted by Berchtold et al. was to synthesise the dihydronaphthalenone, 67, as a starting material, providing the BC ring fragment of the abietane skeleton.\(^4\)
Scheme 1 Synthesis of triptolide (2) via BC→ABC abietane construction

a) NaH, DMF; b) Me₂NH; c) CrO₃, pyridine, CH₂Cl₂; d) neutral Al₂O₃, EtOAc;
e) NaBH₄, EtOH, 2N HCl; f) m-CPBA, CH₂Cl₂; g) Et₃N, CH₂Cl₂; h) 2,4,6-
trimethylpyridine, MeSO₂Cl, DMF; i) H₂, Pd-C, EtOAc; j) CrO₃, AcOH; k) BBr₃,
CH₂Cl₂, 0°C; l) NaBH₄, EtOH; m) NaO₄, MeOH; n) XS m-CPBA, CH₂Cl₂;
o) NaBH₄, EtOH
Scheme 2  Triepoxide construction of the diterpene triepoxides at ring C from laevopimaric acid (74)

a) Rose Bengal, O₂, hv; b) FeSO₄; c) CH₂N₂; d) HCl (cat), Et₂O; e) CrO₃, H₂SO₄, acetone; f) NBS, CCl₄; g) Zn, THF; h) m-CPBA, Na₂CO₃, CH₂Cl₂; i) NaBH₄, MeOH; j) m-CPBA, MeCN; k) (MeCO)₂, PhH, O₂, hv
Scheme 3 Ring A butenolide construction from dehydroabiestic acid (80)

Construction of ring A via annulation of the naphthalenone provided a suitably functionalized tricyclic intermediate, 70, for the construction of the ring C triepoxide system and the butenolide ring (Scheme 1).\(^ {39}\) Annulation was achieved from the addition product of the iodobutyrolactone, 68, to the naphthalenone, 67. Opening of the lactone and oxidation gave the desired intermediate, 69, for the final step of the annulation. Aldol
condensation then yielded the key tricyclic intermediate, 70. Reduction of the aldehyde, acidic hydrolysis and rearrangement of the double bond completed the synthesis of the butenolide ring. Ring C construction was achieved by hydroxylation at C7 and subsequent conversion to the epoxy dienone, 73, by means of periodate oxidation. Further epoxidation gave racemic triptonide (3) while reduction gave a 3:1 mixture of racemic 14-epitriptolide (59) and triptolide (2) respectively.

Tokoroyama et al. devised alternative routes to the C ring system \(^{44}\) (Scheme 2) from laevopimaric acid (74) and to the butenolide ring of triptolide (Scheme 3) and tripdiolide (Scheme 4), \(^{45}\) from dehydroabietic acid (80). The C ring construction (Scheme 2) involved endoperoxide formation and rearrangement to the diepoxide, 75. Double bond manipulations and further epoxidations and reductions yielded the desired triepoxide system, 65, and the epimeric triepoxides, 63 and 64.

Scheme 4 Butenolide construction for the synthesis of tripdiolide (1)

- a) t-BuOK, THF, AcCl;
- b) \(O_2\), Rose Bengal, hu, acetone- MeOH (1:4);
- c)  NBS, \(H_2O\), DMSO;
- d) NaClO\(_2\)
Scheme 5 Total synthesis of triptolide (2) from dehydroabietic acid (80)

a) OsO₄, NaIO₄, AcOH-dioxane-H₂O; b) LDA, HCHO, THF, -78°C; c) MeOC(CH₃)=CH₂, AcOH; d) PhCH₂OCH₂Li, THF, -78°C; e) HCl, THF; f) MeOC(CH₃)=CH₂, AcOH; g) Ac₂O, pyridine; h) HCl, MeOH; i) PCC, CH₂Cl₂; j) o-C₆H₄(NH₂)₂, PhCO₂H, EtOH, HCl; k) NaClO₂, HOSO₂NH₂, dioxane-H₂O; l) H₂, Pd-C, EtOH; m) CrO₃, AcOH-H₂O, 40°C; n) KOH, MeOH-H₂O; o) NaBH₄, EtOH
Scheme 6 Synthesis of triptolide (2) via AB→ABC abietane construction

a) CS₂, 2,6-di-t-Bu-4-Me-C₆H₄OLi, THF, MeI; b) (CH₃)₃S⁺I⁻, NaH, DMSO, -10°C;
c) HCl(aq)-MeOH; d) LDA, HMPA, THF, TBDMSCl; e) CH₂=CCO₂Me, PhH, 65-70°C;
f) 5:1 MeOH-6M HCl; g) MeI, NaH, THF; h) MeLi, THF, -15°C; i) MeSO₂Cl, Et₃N, CH₂Cl₂;
j) Li, NH₃, THF, -78°C; k) m-CPBA, CH₂Cl₂; l) LDA, THF; m) SOCl₂, Et₂O, pyridine, 0°C;
n) KOAc, DMSO, 75°C; o) NaOMe, NaOH; p) (MeO)₂CHNMe₂, xylene, Δ, 4Å sieves;
q) m-CPBA, CH₂Cl₂; r) [(CH₃)₃Si]₂NLi, THF, 0°C; s) 1M HCl

Butenolide construction (Scheme 3) from dehydroabietic acid (80) involved a multistep sequence through the exocyclic olefin, 81, Wittig rearrangement to give 85, and finally SNi' attack on 89 by chloride and closure to the butenolide ring, 91. Synthesis of the tripdiolide analogue, 96, (Scheme 4) consisted of an elimination process via a hydroperoxide to give the diene, 93 and ring closure of the C2 hydroxylated intermediates, 94 and 95 yielding the desired tripdiolide analogue, 96 and its epimeric analogue, 97.
Scheme 7 Biogenetic-type synthesis of triptolide (2)

a) NaH, THF, 0°C; b) Ba(OH)$_2$, H$_2$O-Et$_2$O, 90°C; c) LAH, Et$_2$O, 0°C; d) LiBr, PBr$_3$, collidine, Et$_2$O, -40°C; e) ZnBr, Et$_2$O, 0°C; f) LiH, CH$_3$COCH$_2$CO$_2$Me, DMF, 75°C; g) SnCl$_4$, CH$_2$Cl$_2$, 0°C; h) MeSO$_2$Cl, Et$_3$N, CH$_2$Cl$_2$, 0°C; i) m-CPBA, CH$_2$Cl$_2$; j) LDA, -78°C
van Tamelen and co-workers have devised a number of routes to the total synthesis of triptolide and triptonide. A total synthesis of l-triptonide (3) from dehydroabietic acid (80)\textsuperscript{46} consisted of functionalization of the aromatic ring at C14 in preparation for ring C construction and transformation to the ketone, 100 (Scheme 5). Elaboration to the butenolide, 105, then proceeded via introduction at C3 and C4 of suitably substituted one-carbon moieties, elimination, and ring closure. Introduction of a hydroxyl group at C7 yielded the key intermediate, 72, providing a total synthesis of l-triptonide (3) via the known route to the triepoxide system.

A synthesis of (±)-triptolide, involving fewer steps, comprised construction of ring C onto an appropriate AB fragment (Scheme 6), derived from decalone, 107, and possessing a hydroxyl functionality at C14. An efficient Diels Alder addition to the furan derivative, 109, afforded 110.\textsuperscript{47} Ring A butenolide construction proceeded from the alkene intermediate, 111, via introduction of a hydroxyl group at C3, rearrangement with thionyl chloride and conversion of the C19 allylic chloride to the allylic alcohol, 112. Addition of dimethylformamide dimethylacetal to the allylic alcohol, 112 was followed by a carbene [2,3]-sigmatropic rearrangement to 113. Further elaboration yielded the key intermediate, 36.

A biogenetic-type synthesis of (±)-triptolide consisted of construction of a geranylgeraniol-type intermediate, 120, cyclization to the tricyclic skeleton, 121, and subsequent elaboration of the butenolide ring to give the key intermediate, 36 (Scheme 7).\textsuperscript{48} In one of the key steps, cyclization of the β-keto ester, 120, constituted the first example of a β-keto ester as a cyclization initiator. Appropriate functionality at C3 and C4 as shown in 122 and 123 were introduced for facile conversion to the butenolide ring.

1.4.3 Development of a tissue culture cell line from Tripterygium wilfordii

Isolation and identification of tripdiolide (1) from plant tissue cultures was first reported by Kutney et al.\textsuperscript{49} who isolated the cytotoxic diterpene from tissue suspension cultures of
Tripterygium wilfordii grown in modified B-5 and PRL-4 suspension media. A yield of 0.003% of tripdiolide was reported based on the dry cell weight, and TLC evidence for the presence of triptolide (2) was also reported. A thorough program by Kutney et al., aimed at maximization of tripdiolide yields by way of cell line selection and media optimization resulted in the development of a callus cell line, designated as TRP-4a, from a leaf explant of Tripterygium wilfordii. The callus was initiated on PRI2C0100 agar (PRL-4 medium of Gamborg and Eveleigh without casein hydrolysate) supplemented with indole-3-acetic acid (I) (2 mg/L) and coconut milk (Co) (100 mL/L)) and transferred and maintained on PRD2C0100 agar (PRL-4 medium supplemented with 2,4-dichlorophenoxyacetic acid (D) (2 mg/L) and coconut milk (100 mL/L)). The cell line was chosen for development based on TLC and KB cytotoxicity activity analyses and on growth vigour. Stock suspension cultures of TRP-4a were initiated and maintained in PRD2C0100 broth as a growth medium. Maintenance of the stock culture was carried out by subculturing of a 10% inoculum at 3 week intervals into fresh medium. An extensive study of the growth parameters revealed that resuspension from the growth medium into MSNA0.5K0.5 broth (MS medium of Murashige and Skoog supplemented with naphthaleneacetic acid (NA) (0.5 mg/L) and kinetin (K) (0.5 mg/L)) produced the highest levels of tripdiolide. After 35 days of incubation, tripdiolide levels peaked at 4.0 mg/L, a level 36 times greater than that isolated from the whole plant (based on a dry cell weight of 10 mg/mL). A study on the levels of various nutrients in the medium also showed that tripdiolide production was highest in an MSNA0.5K0.5 medium containing 1650 mg/L of ammonium nitrate, 40 g/L of sucrose, and 880 mg/L of calcium chloride. The method for the production of the TRP-4a cell line and growth of suspension cultures for tripdiolide production is summarized in Figure 2.

Other research in this area has been carried out by Dujack et al. who demonstrated cytotoxic activity of partially purified extracts from tissue cultures of Tripterygium wilfordii which were likely to contain tripdiolide and triptolide. An increase in the biological activity of these extracts was also demonstrated on the incubation of the
cell cultures with pyruvic acid and sodium acetate. Misawa and co-workers showed that the best growth of suspension cultures from their cell lines of *Tripterygium wilfordii* occurred in the MS medium of Murashige and Skoog with 3% sucrose, 1mg/L of kinetin and 0.1mg/L of naphthaleneacetic acid. The highest levels of tripdiolide (96 μg/L) were found after 21 days of cultivation. Tripdiolide levels were shown to increase on the substitution of kinetin with N-phenyl-N'-(2-chloro-4-pyridyl) urea (4-PU-30) and were shown to increase 3-fold on the addition of farnesol to the cell suspension medium.

The development of the TRP-4a cell line for the production of tripdiolide led also to the isolation of several other diterpenoid and triterpenoid compounds. Among those isolated were dehydroabietic acid (80) and the novel diterpene, 124, possessing the 18(4→3) abeo-abietane skeleton.

![Diagram of the development of the TRP-4a cell line of tissue culture from *Tripterygium wilfordii*](image)

**Figure 2** Development of the TRP-4a cell line of tissue culture from *Tripterygium wilfordii*
Other compounds isolated from TRP-4a include the quinone methides celastrol (46) and compounds 125 and 126 of unspecified stereochemistry, oleanolic acid (127), polpunonic acid (55) and β-sitosterol (128).

The structure of the diterpene, 124, was confirmed by synthesis according to Scheme 8. The ketone, 129, was synthesised from dehydroabietic acid (80) following the procedure of Huffman. Introduction of the C3 functionality was achieved by hydrocyanation and elimination followed by reduction, oxidation and esterification. Hydroxylation at C15 was achieved photochemically in the presence of oxygen.

a) Pb(OAc)$_4$, PhH, pyridine, $\Delta$; b) BF$_3$-LAH, NaOH, H$_2$O$_2$; c) PCC; d) KCN, 18-crown-6, DMSO-DMF; e) SOCl$_2$, pyridine; f) NaOEt, EtOH; g) DIBAL-H, hexane, 0°C; h) NaClO$_2$, resorcinol, dioxane-H$_2$O-1N HCl; i) CH$_2$N$_2$; j) O$_2$, hv
1.5 Recent interest in *Tripterygium wilfordii*

1.5.1 Clinical uses

The past 20 or more years has seen the increasing clinical use, by Western-trained physicians in China, of extracts from the plant. Various disorders have been treated ranging from rheumatoid arthritis and ankylosing spondilitis through a variety of skin disorders including Behcet's disease, psoriatic arthritis, pustular psoriasis, systemic lupus erythematosus, allergic angiitis, Sweet's syndrome, lepra reactions, etc. The extract, used for these disorders, is found to be more potent than the conventional non-steroidal antirheumatic agents such as salicylates, indomethacin and phenylbutazone, and can be substituted for corticosteroids in some skin diseases and in some patients who are steroid-dependent or who have contraindications to steroids. Its therapeutic effectiveness is believed to be related to its antiinflammatory and immunosuppressive effects.

The preparations which have been used in these treatments are extracts from the dried root xylem of the plant (collected in summer or early autumn) and fall into two main categories:

1) The crude extract. A daily dose of the root cuttings of 15-25 g is extracted with boiling water and concentrated. An alcoholic extract is also used with 2-4 g of the root cuttings.

2) The refined extracts. The two types of refined extract are the "multi-glycosides" (GTW) and the alkaloids. The alkaloid extract is not used clinically because it has no definite therapeutic effect and shows severe side effects. GTW is prepared by extraction of the root xylem with water and chloroform followed by column
10 mg of the GTW and a recommended dose for treatment of rheumatoid arthritis and skin disorders is 60-90 mg per day.

1.5.2 Pharmacological studies

Preliminary pharmacological studies revealed that rats given the crude extracts hypodermically for up to 68 days showed degenerative changes in the heart, kidney, liver and spleen, haemorrhagic foci in the spleen, liver and kidney, and congestion of the central nervous system and the gastrointestinal tract. A later study showed that alcoholic extracts of the root xylem depressed the blood pressure in rabbits, inhibited respiration, excited the atrium in vitro, disordered the heartbeat and stimulated unstriated muscle. Although these effects were observed, they were deemed to be very moderate at clinical doses with the toxicity being mainly due to the effect on the heart.

Immunological studies showed that extracts of Tripterygium wilfordii had an immunosuppressive action on humoral and cell-mediated immunity. GTW was shown to inhibit antibody production in antigen-bound and antibody-secreting cells in rats. Inhibition of the OT skin test, RE rosette formation and lymphocyte transformation were also exhibited in guinea pigs.

A comparison of the pharmacological effects of GTW with the alkaloids and the crude extract showed that GTW is the main antiinflammatory constituent of Tripterygium wilfordii. It has been pointed out that the term "multi-glycoside" (GTW) is inappropriate for this refined extract. The term only implies that some glycosides are present in the preparation in addition to other constituents (possibly pentacyclic triterpenes and some diterpenes), but are not necessarily the active constituents.

1.5.3 Side effects

At the regular clinical dosage levels, the main side effects are found to be gastrointestinal disturbances including nausea, vomiting, anorexia, epigastric burning
sensation, xerostomia, diarrhoea and constipation. The side effects are not severe and usually subside without discontinuation of the treatment. Leukopenia and thrombocytopenia were observed in a very few cases with full recovery on cessation of treatment. GTW had fewer and less severe side effects than the crude extract preparation of the drug. Other side effects include menstrual disturbances, oligospermia, azoospermia, and a decrease in the size of the testis.

1.5.4 *Tripterygium wilfordii* in antifertility studies

The observed effects on the reproductive system, mentioned above, have stimulated a great deal of research into the effect of GTW, particularly on the male reproductive system, and its possible use as a birth control agent. Zheng et al. carried out further toxicity studies on GTW with emphasis on the effects on the male reproductive system. Their results showed that GTW caused damage of the seminiferous epidermis of the testis and inhibited spermatogonium mitosis in experimental animals which resulted in reduction or absence of reproductive cells of different stages. Subthreshold doses of GTW caused a reduction of the pregnancy rate and also sterility of mice. The observed effects were found to be reversible on discontinuation of administration of GTW. It was also observed that the effect of GTW on the testis is extremely similar to that of gossypol and that the safety limit of GTW is much higher than the crude extract.

An independent study by Qian et al., on male and female rats, showed that at doses of 10 mg/kg per day of GTW, male rats became infertile at the end of the eighth week of treatment. At these dose levels (much lower than in the previous study), no damage to the seminiferous tubules was observed, and no decrease in testosterone levels was observed. In female rats, no effect on fertility was observed. In men, infertility was observed at doses of one-third of that recommended for treatment of arthritis and skin disorders. The libido and potency of treated patients were not affected. Reversibility of infertility was reported on cessation of the treatment. Infertility was observed to be
caused by a decrease in density of spermatozoa and a decrease in the motility of the spermatozoa to almost nil. The antifertility effects have been reviewed.\textsuperscript{59,69}

Because of the promising antiinflammatory, immunosuppressive and antifertility activities of GTW, much work is under way to isolate and identify the active principles. Preliminary screening of 8 unspecified components (designated $T_1$-$T_8$) from GTW, revealed that antiinflammatory-immunosuppressive activities were inseparable from antifertility activity and that antiinflammatory and immunosuppressive activity overlapped between components $T_2$-$T_4$ and deviated in components $T_1$ and $T_5$.\textsuperscript{70} Five monomers were also screened, only one of which showed immunosuppressant and antifertility activities. All 5 monomers exhibited antiinflammatory activity in mice.\textsuperscript{71} The apparent inseparability of immunosuppressant activity and antifertility activity in these components would seem to limit the use of GTW in fertility regulation. However, many more components have yet to be investigated and research is continuing in this area.

1.6 Objectives of the present investigation

In view of the current interest and intensive research in China towards discovering the active principles from \textit{Tripterygium wilfordii} responsible for the immunosuppressive, antiinflammatory and antifertility activities, the present investigation is aimed at a more comprehensive investigation of the metabolites present in the tissue culture cell line TRP-4a. The similarity in the spectrum of metabolites thus far isolated from TRP-4a cultures to those isolated from the whole plant, suggests that TRP-4a cultures may possess similar biological activities. Preliminary screening of crude extracts and subsequent isolated metabolites for immunosuppressive and antifertility activities is anticipated in an effort to establish any of the above activities and to identify the active components from TRP-4a tissue cultures.

The interesting biological activities of the diterpene triepoxides, discovered by Kupchan and other research groups in China, and their sparse availability, make them
suitable targets for biosynthetic studies. The present investigation is also directed at a preliminary investigation into the biosynthetic pathway to the diterpene triepoxides, in order to determine the late stage precursors involved in their biosynthesis.
2 RESULTS AND DISCUSSION

PART A

ISOLATION AND STRUCTURE ELUCIDATION OF THE MAJOR
METABOLITES FROM TISSUE CULTURES OF Tripterygium wilfordii

2.1 TRP-4a cell production and preliminary screening for biological activity

In order to isolate sufficient quantities of extracts (and later, metabolites) from the tissue culture cells (TRP-4a cell line) for biological screening, it was necessary to produce large scale quantities of the suspension culture. Batch fermentations were carried out on 10-17 L scale in glass or in steel air-lift fermentors. Suspension cultures were grown according to the procedure outlined in Planta Medica 51 (see also Figure 2). Stock suspension cultures maintained in PRD₂Co₁₀₀ growth medium were resuspended in MSNA₀.₅K₀.₅ production medium and growth conditions (27°C, without light, agitation) were maintained until the refractive index of the suspension decreased to a value of approximately 1.3334. At this point, the cells were considered to have stopped growing (onset of stationary phase) and were harvested. The cells were separated from the spent medium by filtration and were extracted separately.

For the purposes of preliminary screening for biological activity of extracts from TRP-4a cultures, an initial 15 L batch of cell suspension culture was grown. Stationary phase was reached 33 days after resuspension in the production medium (MSNA₀.₅K₀.₅) and the culture was harvested. This batch was designated as TRP#221.

A general extraction procedure was developed in which the cells and the spent medium were extracted separately (Figures 3 and 4). Extraction of the cells was carried out using ethyl acetate and methanol. The cells were homogenized in ethyl acetate, followed by filtration through celite, and the cell residue was washed with ethyl acetate.
The aqueous filtrate was separated, extracted with ethyl acetate and the organic layers were combined. Solvent removal yielded the ethyl acetate extract. Sonication of the cell residue in methanol, followed by filtration through celite, washing with methanol and solvent removal constituted the methanol extract.

**Figure 3 Extraction procedure for the TRP-4a cells**
**Figure 4** Extraction procedure for the TRP-4a spent medium
The freeze-dried spent medium was reconstituted with water and extracted with ethyl acetate. The methanol extract was obtained by evaporation of the water, drying the residue, sonication of the residue in methanol, and filtration.

Samples of the extracts were submitted for preliminary screening of biological activity to laboratories in Shanghai and Nanjing, People's Republic of China. Assays for immunosuppressive activity and antifertility activity were carried out. Immunosuppressive assays were performed in vivo using C57/BL inbred mice. In vitro tests were carried out for the inhibition of proliferation of lymphocytes (by the $^3$H-thymidine incorporation test) and for the inhibition of antibody formation (by the serum haemolysin HC50 unit test and spleen plaque-forming cells count). Antifertility assays were performed in vivo and tests were carried out for antispermatic activity.

The results of the preliminary screening of the crude extracts from TRP#221 showed that the ethyl acetate extracts had both immunosuppressive activities and antifertility activity while the methanol extracts were inactive in both cases.

<table>
<thead>
<tr>
<th>Batch No.</th>
<th>Culture vol. (L)</th>
<th>Incubation time (days)</th>
<th>R.I. on harvesting</th>
</tr>
</thead>
<tbody>
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</tr>
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<td>1.3338</td>
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<tr>
<td>TRP#225</td>
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Table 1 Volumes and growth times for initial batches of TRP-4a suspension cultures for preliminary biological screening
<table>
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<th>Batch No.</th>
<th>Wet wt. cells (kg)</th>
<th>Spent medium vol. (L)</th>
<th>EtOAc Extract (g)</th>
<th>MeOH Extract (g)</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td>Cells</td>
<td>Spent medium</td>
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<td>TRP#224</td>
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<td>0.10</td>
</tr>
</tbody>
</table>

Table 2 Weights of extracts from TRP-4a suspension cultures

Three further batches of TRP-4a cell suspension cultures were grown and harvested in the same way (Table 1). Extraction of the cells and the spent medium was carried out as above to give ethyl acetate and methanol extracts (Table 2). The ethyl acetate extracts were generally dark brown solids, while the methanol extracts were dark brown gummy residues.

2.2 Partial separation and biological screening of the ethyl acetate extracts from TRP-4a cells and spent medium

The ethyl acetate extracts from batches TRP#222, TRP#224 and TRP#225 were combined to give 3.73 g of cell extract and 1.71 g of spent medium extract. The methanol

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1 At this time, the results of the preliminary biological assays were available, which showed the methanol extracts to be inactive (vide supra). A methanol extraction for this batch was, therefore, not carried out.
extracts were not further investigated. The ethyl acetate extracts were subjected to partial separation in order to give crude fractions for further preliminary biological screening.

Partial separation of the cell extract was carried out by vacuum liquid chromatography (VLC) as described by Pelletier et al. and J.C. Coll et al. The "column" (sintered glass funnel) was packed with silica gel (Merck silica gel 60G) and a stepwise elution was carried out using: i) hexanes-ethyl acetate 4:1; ii) hexanes-ethyl acetate 3:1; iii) hexanes-ethyl acetate 2:1; iv) hexanes-ethyl acetate 1:1 and v) ethyl acetate. The column was finally flushed with ethyl acetate-methanol 1:1 to remove the polar material. Eight crude fractions containing complex mixtures of metabolites were collected. The hexanes-ethyl acetate 4:1 elution afforded an oily fraction containing fatty acid-like compounds (0.38 g). Fractions 2 to 7 were yellow to orangey-brown solids containing sterols, diterpenes, and triterpenes (0.39 g, 0.27 g, 0.27 g, 0.51 g, 0.29 g, and 0.18 g respectively). Fraction 8 was a dark brown solid (1.22 g) containing the polar material.

Biological screening of the fractions showed that fractions 6 and 7 were active in antifertility and in inhibition of lymphocyte proliferation. Fractions 6 and 7 were crude fractions containing triterpenes as the major constituents.

Partial separation of the spent medium extract was carried out by VLC using silica gel and stepwise elution from: i) hexanes; ii) hexanes-ethyl acetate 19:1; iii) hexanes-ethyl acetate 9:1; iv) hexanes-ethyl acetate 4:1; v) hexanes-ethyl acetate 1:1 and vi) ethyl acetate. The column was finally flushed with ethyl acetate-methanol 1:1 to remove the polar material. Ten fractions containing complex mixtures of compounds were collected. Fractions 1 to 5 consisted of complex mixtures of diterpenes, sterols, etc. (4.32 mg, 23.89 mg, 14.28 mg, 60.64 mg, and 128.52 mg respectively), and were all yellow-brown solids. Fraction 6 consisted of triptolide (2) and a number of triterpenes as the major constituents (206.21 mg). Fraction 7 consisted of tripdiolide (1) and triterpenes as
the major constituents (204.75 mg). Both fractions 6 and 7 were eluted with ethyl acetate and were yellow-brown solids. Elution with ethyl acetate-methanol 1:1 gave fractions 8, 9 and 10 (brown solids, 52.43 mg, 303.69 mg and 76.54 mg respectively). Fraction 8 contained a small amount of triterpene compounds and other polar material, while fractions 9 and 10 contained only the polar material.

Biological screening of the spent medium fractions showed that fractions 6 and 7 possessed the highest antifertility activity while fraction 8 showed moderate activity. Fractions 6 and 7 also demonstrated inhibitory activity against lymphocyte proliferation.

2.3 Isolation and structure elucidation of terpenoid metabolites from the TRP-4a tissue cultures

Evidence from the biological screening of crude fractions from the TRP-4a cultures points towards the possibility that the active component or components are the diterpene triepoxides and/or triterpenoid compounds. Efforts were first directed at isolating the metabolites from the fractions exhibiting biological activity.

2.3.1 Growth and extraction of further batches of TRP-4a cultures

Six batches of TRP-4a suspension culture were grown as described previously, ranging from 10 to 17 L in quantity and over periods of 25 to 39 days (Table 3). On harvesting, the cells and spent medium were separated. The cells were frozen until the time of extraction, and the spent medium was freeze-dried until reduced to a volume of 1.4 L and then frozen until the time of extraction. Extraction of the combined cells and the spent medium was carried out as previously described to obtain ethyl acetate extracts. Methanol extraction was omitted.
Extraction of the cells with ethyl acetate yielded 13.08 g of a dark orange solid, while extraction of the spent medium yielded 4.73 g of a dark orangey-brown solid. TLC comparison demonstrated the presence in the extracts, of the same metabolites found in the biologically active fractions. The TLC comparisons were carried out using ethyl acetate, chloroform-methanol-acetic acid 95:5:1, and benzene-methanol- acetic acid 90:5:5 (double development) as the eluting solvents.

<table>
<thead>
<tr>
<th>Batch No.</th>
<th>Culture vol. (L)</th>
<th>Incubation time (days)</th>
<th>R.I.</th>
<th>Spent medium vol. (L)</th>
<th>Wet wt. cells (kg)</th>
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</table>

Table 3 Volumes and growth times for further batches of TRP-4a suspension cultures
2.3.2 Isolation and structure elucidation of the metabolites from the cell extract of the TRP-4a suspension cultures

i) Triterpene acids from the biologically active fractions

The polar material was removed from the cell extract by filtration through silica gel using ethyl acetate as the solvent and yielding a fraction of 9.04 g, as an orange solid, from an original extract of 13.08 g. An initial attempt at the separation of the metabolites found in the biologically active fractions was carried out on a portion of the crude extract involving VLC and preparative TLC of the crude fractions (Method A). An improved procedure involved VLC and column chromatography (Method B).

Method A: VLC separation of the crude extract (3.01 g) by stepwise elution (30 mL fractions were collected) using: i) benzene; ii) benzene-acetone 4:1; iii) benzene-acetone 3:1; iv) benzene-acetone 2:1 and rapid elution with ethyl acetate yielded 10 crude fractions. Elution with benzene yielded 2 clear oily fractions (fractions 1 and 2) while elution with benzene-acetone 4:1 yielded a dark orange solid (fraction 3). Fraction 4, also eluted with benzene-acetone 4:1 was shown to be oleanolic acid (127) (432.26 mg). Further elution with benzene-acetone 4:1 yielded fractions 5 to 7 containing the triterpenes 137, 138, 139 and 51 (compounds present in the previous biologically active fractions). Fractions 8 and 9 were eluted with benzene-acetone 3:1 and 2:1 while fraction 10 was eluted with ethyl acetate. Fraction 8 contained further quantities of 139 and 51 and fractions 9 and 10 contained further quantities of 51. Preparative TLC purification of fractions 5 to 8 (methylene chloride-methanol-acetic acid 100:2:1) yielded the pure triterpenes 137 (27.25 mg), 138 (65.77 mg), 139 (20.85 mg) and 51. Fractions 9 and 10 were washed with methanol to give further quantities of 51 (178.93 mg).
Method B: Separation of the remaining cell extract (5.83 mg) as above by VLC and stepwise elution with the same benzene-acetone mixtures, gave 10 crude fractions. Fraction 1 was a colourless oily fraction consisting of fatty acid-like compounds. Fraction 2 was a dark orange solid while fraction 3 was oleanolic acid (127) and a small quantity of the triterpene 137. Fractions 4 to 10 contained the triterpenes 137, 138, 139 and 51. Purification of fractions 3 to 8 by column chromatography (methylene chloride- methanol-acetic acid 100:1:1 and 100:2:1) and washing of fractions 9 and 10 with methanol gave oleanolic acid (127, 101.97 mg) and the triterpenes 137 (55.80 mg), 138 (289.43 mg), 139 (181.17 mg) and 51 (444.99 mg).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Quantity (mg)</th>
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<tr>
<td>137</td>
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</tr>
<tr>
<td>138</td>
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<tr>
<td>51</td>
<td>623.92</td>
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Table 4 Quantities of the triterpenes 137, 138, 139 and 51 isolated from the cell extract
The triterpene 137 was isolated as optically active ($\left[\alpha\right]_D^{22} +92.9$) colourless prisms (mp 268-270°C) with a molecular formula of $C_{30}H_{46}O_4$ (high resolution mass spectrometry). Its IR spectrum shows a hydroxyl group (3618 cm$^{-1}$), a broad band at 2640 cm$^{-1}$ and a carbonyl band at 1698 cm$^{-1}$ indicating the presence of a carboxylic acid group and also possibly a ketone group. Strong evidence for an oleanene-type triterpene skeleton is given in the fragmentation pattern of the mass spectrum of 137. A retro Diels-Alder (RDA) fragmentation is characteristic of the oleanene skeleton where the DE ring fragment maintains the positive charge. The AB ring fragment also maintains the positive charge, but to a much lesser extent.$^{74}$ This fragmentation places the double bond at C12.
In 137, a fragment peak is observed at m/z 264 corresponding to the RDA fragmentation. The mass of the RDA fragment suggests that the carboxylic acid and hydroxyl functionalities are located in the DE fragment, and that the ketone, therefore, is in the AB fragment of the skeleton (m/z 205). Loss of water from the RDA fragment at m/z 264, giving rise to a fragment ion at m/z 246 provides further evidence for the location of the OH in the DE fragment. Weak fragment ions at m/z 426 and 424 corresponding to M⁺- CO₂, and M⁺- HCO₂H point to the preclusion of the carboxyl group being positioned at C14 or C17 since angular carboxyl groups are more easily lost, giving rise to much stronger peaks of this nature ⁷⁵ (cf. oleanolic acid, *vide infra*). The carboxyl group must therefore be located at C20 in either the α or β orientation. The observation of the above RDA fragmentation pattern also suggests that the ketone functionality is not at C1 or C7 since a ketone in either of these positions would result in McLafferty rearrangements, leading to a different fragmentation pattern. Based on this evidence and on the ¹H NMR coupling pattern (*vide infra*), the ketone must be positioned at C3.
The $^1$H NMR spectrum reveals seven sharp methyl group signals ($\delta$ 0.91, 1.04, 1.07, 1.09, 1.11, 1.15 and 1.41) and a signal at $\delta$ 5.33 (1H, t), characteristic of oleanene-type triterpenes. A signal is observed at $\delta$ 3.58 (1H, dd, $J = 3.5$, 7.0 Hz) providing evidence for an axial secondary hydroxyl group, with two adjacent hydrogens, attached to
a 6-membered ring. Signals at δ 2.39 (1H, ddd, J = 4.0, 6.0, 16.0 Hz) and δ 2.55 (1H, ddd, J = 4.0, 11.0, 16.0 Hz) show the presence of 2 geminal protons (J = 16.0 Hz) α to a carbonyl and with two neighbouring hydrogens. A sharp singlet at δ 3.50 disappears on addition of D₂O, providing further evidence for a hydroxyl group. The ¹³C NMR spectrum shows signals at δ 75.4, 122.9, 144.2, 181.6 and 217.8 corresponding to C-OH, 2 alkene carbons, carboxylic acid and ketone signals respectively. The APT spectrum points out the seven methyl group signals (δ 15.4, 17.2, 21.1, 21.8, 25.3, 25.4, and 26.7) and that the OH is attached to a methine carbon (δ 75.4), since these signals are inverted in the spectrum (ie. carbons with 1 or 3 hydrogens attached give rise to inverted signals in the APT spectrum). The alkene carbon at δ 122.9 is shown to have one proton attached, while the alkene carbon at δ 144.2 is fully substituted (the signal is not inverted in the APT spectrum). The remaining 5 degrees of unsaturation, as given by the molecular formula C₃₀H₄₆O₄, are accounted for by a pentacyclic carbon skeleton. The position of the hydroxyl group and the relative stereochemistry of the molecule were confirmed by chemical correlation with the triterpene, 51 (Scheme 9, vide infra).

The triterpene 138, was isolated as optically active ([α]₂₅ +84.4) colourless prisms (mp 289-290°C) with a molecular formula of C₃₀H₄₆O₄. Its IR spectrum shows hydroxyl (3615 cm⁻¹), carboxylic acid (2630 cm⁻¹) and carbonyl (1698 cm⁻¹) groups. In the mass spectrum, the molecular ion peak (m/z 470) and the RDA fragment ion (m/z 264) are very weak, being much less intense than those observed for 137. Instead, strong peaks are observed at m/z 452 (M⁺ - H₂O) and 246 (base peak M⁺ - RDA -H₂O) suggesting that water is lost more readily from this structure than from 137. A possible inference from this observation might be lactonization, which would place the OH at C22α (molecular model studies indicate that the C22α position is equatorial assuming a chair conformation of ring E) and the carboxyl group at C20α (no M⁺ - 44, 45 or 46 peaks are observed in the mass spectrum). Lactonization is corroborated by the observation that
the fragment ion at m/z 246 is relatively stable (base peak). A ketone at C3 is supported by an RDA fragment ion at m/z 205.

The $^1$H NMR spectrum is very similar to that of 137, except that at $\delta$ 3.60 (1H, dd, $J = 4.0$, 12.0 Hz), an equatorial OH functionality is indicated. Two protons $\alpha$ to a ketone are present at $\delta$ 2.38 (1H, ddd, $J = 4.0$, 6.0, 16.0 Hz) and $\delta$ 2.55 (1H, ddd, $J = 4.0$, 11.0, 16.0 Hz) along with the olefinic proton at $\delta$ 5.29 (1H, t) and the seven methyl group singlets at $\delta$ 1.02, 1.04, 1.07, 1.08, 1.10, 1.18, and 1.29. The hydroxyl proton is not observed but may
be buried in the aliphatic region of the spectrum. The $^{13}$C NMR spectrum shows the presence of C-OH at $\delta$ 75.3, alkene carbons at $\delta$ 123.1 and 142.6, carboxyl at $\delta$ 183.3 and ketone at $\delta$ 217.8. The APT spectrum confirms that the hydroxyl group is secondary, showing an inverted CHOH signal, that the alkene is trisubstituted (an inverted signal is observed at $\delta$ 123.1) and that seven methyl groups are present in the structure.

Further support for the structure of triterpene 138 is given in a 2D $^1$H-$^1$H correlated NMR spectrum (COSY, Figure 5), where the cross peaks in the spectrum indicate coupling between adjacent protons (scalar coupling). The cross peak for the AX$_2$ system H12 - H11 is very prominent and from this can be traced the coupling of the H11 protons to the H9 proton. Also evident are the cross peaks from the proton geminal to the OH to the adjacent protons (22β/21β, and 22β/21α). From this can be traced the geminal 21β/21α cross peak. Further support for a C3 ketone comes from the appearance of the cross peaks 2α/1, 2α/1', 2β/1, 2β/1', 2α/2β, and 1/1'. The remainder of the crowded aliphatic region below $\delta$ 2.60 is difficult to discern. However, tentative assignments of the cross peaks have been made (Figure 6). Confirmation of the structure and relative stereochemistry of 138 was provided by chemical correlation with 51 (Scheme 9).

The dihydroxy triterpene, 139, was isolated as optically active ([α]$_D^{22}$ +55.0) colourless prisms (mp 281-283°C) with a molecular formula of C$_{30}$H$_{48}$O$_4$. Its IR spectrum shows two OH bands (3467, 3422 cm$^{-1}$), carboxylic acid (2640 cm$^{-1}$) and carbonyl (1698 cm$^{-1}$) absorptions. The mass spectrum of 139 is similar to that of 137: the molecular ion peak at m/z 472 shows a significant RDA fragmentation to give a peak at m/z 264 which shows a further loss of water to give a fragment ion at m/z 246. A base peak at m/z 217 is also observed, due to further loss of C$_2$H$_7$O from the RDA fragment ion (this peak is also observed for 137 but is much less intense). No significant peak is observed for loss of a carboxyl group (M$^+$- 45) therefore placing the carboxyl group at C20. The RDA fragmentation pattern places one hydroxyl in the AB ring fragment (m/z 207) and the other in the DE ring fragment (m/z 264).
Figure 5 COSY spectrum of 138
Figure 6 Expanded COSY spectrum of 138
Its $^1$H NMR spectrum (C$_5$D$_5$N) shows seven methyl group singlets characteristic of the oleanene skeleton ($\delta$ 0.76, 0.85 (6H), 1.02, 1.03, 1.08, and 1.63), 2 signals at $\delta$ 3.22 (1H, dd, $J = 6.0$, 9.5 Hz) and $\delta$ 3.80 (1H, brd, $J = 4.5$Hz) tentatively assigned to protons at C3$\alpha$ and C22$\beta$ geminal to equatorial and axial OH groups respectively, and an olefin signal at $\delta$ 5.21 (1H, t). In the $^{13}$C NMR spectrum, two carbons bearing OH are evident at $\delta$ 75.3 and 77.9, and the two alkene carbons are clearly seen at $\delta$ 123.1 and 144.2. The carboxyl carbon is seen as a weak signal at $\delta$ 181.0. The APT spectrum indicates quite clearly the presence of seven methyl groups at $\delta$ 15.8, 16.6, 17.2, 21.0, 24.9, 25.5 and 28.7. The two hydroxyl bearing carbons give inverted signals (indicating secondary hydroxyl groups), and the alkene signal at $\delta$ 123.1 is inverted indicating the presence of the trisubstituted alkene at C12. The exact locations of the hydroxyl groups were shown, by chemical correlation with 51 (Scheme 9), to be C3$\beta$ and C22$\beta$.

The dihydroxy triterpene, 51, was isolated as optically active ($[\alpha]_{D}^{22} +93.5$) colourless prisms (mp 293-298°C) with a molecular formula of C$_{30}$H$_{48}$O$_{4}$. Its IR spectrum shows two hydroxyl groups (3475, 3387 cm$^{-1}$) a carboxylic acid (2550 cm$^{-1}$), and a carbonyl (1698 cm$^{-1}$) absorptions. The mass spectrum of 51 is similar to that of 138, exhibiting a weak molecular ion peak at m/z 472 due to loss of water (m/z 454 and 436). No M$^+$- CO$_2$H is observed, and the RDA fragment ion at m/z 264, undergoes facile loss of water to give a base peak at m/z 246. The above observations indicate that the carboxyl group is at C20 with one hydroxyl located in the AB fragment (m/z 207) while the other is located in the DE fragment. The $^1$H NMR spectrum (C$_5$D$_5$N) reveals seven methyl group singlets ($\delta$ 0.98, 1.03, 1.04, 1.23, 1.28, 1.37 and 1.58) and two signals at $\delta$ 3.42 (1H, dd, $J = 6.0$, 10.0 Hz) and $\delta$ 4.01 (1H, dd, $J = 5.5$, 13.0 Hz) corresponding to protons geminal to two equatorial hydroxyl groups. An olefinic proton is also observed at $\delta$ 5.38 (1H, t). The $^{13}$C NMR spectrum exhibits hydroxyl-bearing carbons at $\delta$ 74.7 and 78.0, alkene carbons at $\delta$ 123.1 and 144.2 and a carboxyl carbon at 181.1.
Figure 7  COSY spectrum of 51
Figure 8  Computer generated X-ray structure of 51
Scheme 9 Chemical correlation of the triterpenes, 137, 138, 139, and 51
The APT spectrum shows the presence of seven methyl group signals at δ 15.8, 16.6, 17.0, 21.4, 25.4, 26.3 and 28.7, two secondary hydroxyl groups (δ74.7 and 78.0) and a trisubstituted double bond (the signal at δ123.1 is inverted while the signal at δ144.2 is not).

A 2D ¹H-¹H COSY spectrum of 51 (Figure 7) exhibits the cross peaks 12/11,11' and 11,11'/9 as seen for the triterpene 138. The 22β proton at δ 4.01 shows cross peaks to two other protons (22β/21β and 22β/21α) which show geminal coupling to each other. The 3α proton at δ 3.42 shows cross peaks to two other protons (3α/2,2') which themselves show evidence of further coupling but which is difficult to discern due to crowding around the diagonal.

The structure and relative stereochemistry of 51 were confirmed by a single crystal X-ray analysis (Figure 8). The computer-generated X-ray structure depicts ring E in a chair conformation placing the C20α carboxylic acid and the C22α hydroxyl groups in equatorial orientations. The C3 hydroxyl group is also shown to be equatorial (C3β) as predicted from the ¹H NMR spectrum. The crystal exists as the 1:1 methanol solvate, with the methanol oxygen coordinating with all three hydroxyls of the triterpene.

The triterpenes 137 and 138 were reduced with sodium borohydride (Scheme 9). Triterpene 137 yielded a compound with identical TLC, mp, IR, MS and ¹H NMR spectra to 139. Triterpene 138 yielded a compound with identical TLC, mp, IR, MS and ¹H NMR spectra to 51. Esterification of 51 followed by oxidation with pyridinium chlorochromate yielded the diketone, 144 as an optically active ([α]_D^22 +5.7) white solid (mp 180.5-182°C) with a molecular formula of C₃₁H₄₆O₄. Its IR spectrum shows carbonyls at 1721 and 1698 cm⁻¹ and C-O absorption at 1218 cm⁻¹. The ¹H NMR spectrum shows four signals adjacent to carbonyl at δ 2.39 (1H, ddd, C2αH), 2.48 (1H, d, C21βH), 2.56 (1H, ddd, C2βH) and 2.99 (1H, d, C21αH). Esterification and oxidation of 139 in the same manner, yielded a compound with identical TLC, mp, IR, MS and ¹H NMR spectra to 144.
The spectral data and chemical interconversions show that the structure of 137 is 22β-hydroxy-3-oxoolean-12-en-29-oic acid, 138 is 22α-hydroxy-3-oxoolean-12-en-29-oic acid, 139 is 3β,22β-dihydroxyolean-12-en-29-oic acid and that 51 is 3β,22α-dihydroxyolean-12-en-29-oic acid.

The mass spectral data for compounds 137, 138, 139 and 51 are summarized and compared in Table 5, while the 1H NMR and 13C NMR data are summarized and compared in Tables 6 and 7 respectively.

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Table 5 Mass spectral comparison of triterpenes 137, 138, 139 and 51
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Table 6 Comparison of the $^1$H NMR spectra of the triterpenes 137, 138, 139 and 51

¹ CDCl$_3$

² C$_2$D$_3$N
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Table 7 Comparison of the $^{13}$C NMR spectra of the triterpenes 137, 138, 139 and 51

$^1$ CDCl$_3$

$^2$ C$_3$D$_2$N
The compounds 137, 138 and 139 are new triterpenes. Compound 51 is isolated for the first time from the TRP-4a cell line suspension cultures of *Tripterygium wilfordii*, and has been isolated previously from the whole plant.\(^{32,33}\) Compound 51 is identical with maytenfolic acid \(^{77}\) and abrusgenic acid. \(^{78}\) Maytenfolic acid was found to exhibit significant antileukaemic activity.

Oleanolic acid (127) was isolated as optically active (\([\alpha]_D^{22} +58.1\)) colourless prisms (mp 268-269°C) and its IR, MS and \(^1\)H NMR spectra are identical with those in the literature.\(^{57}\) The mass spectrum shows a molecular ion peak at m/z 456 and an RDA fragment ion peak at m/z 248. Also evident is a relatively strong \(M^+ - 45\) (\(M^+ - \text{CO}_2\text{H}\)) peak at m/z 411 and RDA -45 at m/z 203 corresponding to loss of the angular C17 carboxylic acid. The \(^{13}\)C NMR spectrum shows C3 (CHOH) at \(\delta 78.2\), two alkene carbons at \(\delta 123.2\) and 144.7 (C12 and C13 respectively) and carboxyl (C28) at \(\delta 181.4\). The APT spectrum reveals seven methyl group signals at \(\delta 16.1, 17.0, 17.4, 20.4, 26.5, 28.8\) and 29.1.

ii) Isolation of the major metabolites from further fractions of the cell extract

Fraction 3 (1.17 g) from Method A and fraction 2 (2.38 g) from Method B above, the dark orange fractions, were combined and subjected to column chromatography by stepwise elution using: i) hexanes; ii) hexanes-ethyl acetate 9:1; iii) hexanes-ethyl acetate 4:1; iv) hexanes-ethyl acetate 3:1; v) hexanes-ethyl acetate 2:1 and iv) ethyl acetate. The first two fractions, A and B yielded complex mixtures of non-polar minor components. Fractions C and D yielded triterpenes and an abietane-type diterpene. Further elution gave two fractions (E and F) of \(\beta\)-sitosterol (128, 553.25 mg) and 3 fractions, G, H and I, containing complex mixtures of minor components. Fractions J and K contained orange-coloured quinone methides and polpunonic acid (55) and a final
fraction, L, yielded further quantities of oleanolic acid (127, 564.65 mg). β-sitosterol (128) was compared with an authentic sample (isolated by this research group) and was found to be identical by TLC, mp, IR, MS and $^1$H NMR spectroscopy.

Fractions C and D:

Fractions C and D were further purified by preparative TLC (methylene chloride-ethyl acetate 19:1 and methylene chloride-hexanes 1:1) to give an inseparable mixture of what appears to be, from the spectral data, α- and β-amyrin (145 and 146 respectively, 121.81 mg), and the abietane-type diterpene, 147 (49.68 mg).

The triterpene mixture, 145 and 146, was isolated as a white solid with an IR spectrum showing hydroxyl (3445 cm$^{-1}$) and alkene (1638 cm$^{-1}$) groups. The mass
spectrum exhibits a molecular ion peak at m/z 426 which at high resolution gives a molecular formula of C_{30}H_{50}O. A base peak at m/z 218 is indicative of the RDA fragmentation characteristic of the oleanene (and ursene in the case of 145) type skeleton. A minor fragmentation ion at m/z 207 corresponds to the AB fragment carrying the positive charge following the RDA fragmentation. The mass of this fragment ion places the OH in the AB fragment. Its $^1$H NMR spectrum is quite complex, exhibiting a series of signals corresponding to tertiary methyl groups and a signal at δ 0.81 which appears to be a doublet corresponding to two secondary methyl groups. Two signals corresponding to secondary hydroxyl groups (at C3) appear at δ 3.23 and 3.48 as multiplets and olefinic signals appear at δ 5.11 and 5.19 (C12 double bond). In the $^{13}$C NMR spectrum, signals appear at δ 78.7 and 79.0 corresponding to hydroxyl-bearing carbons (C3). Signals are also observed at δ 117.8 and 121.7 (C12) and at δ 145.3 and 145.9 (C13). Much weaker signals appear in both the $^1$H and $^{13}$C NMR spectra indicating the presence of a minor impurity in the mixture.

The abietane-type diterpene, 147, was isolated as optically active ([α]_D^{22} +44.4) colourless needles (mp 157-158°C) with a molecular formula of C_{21}H_{32}O_2. Its IR spectrum has absorption bands for hydroxyl (3620 cm⁻¹) and benzene ring (1488 cm⁻¹) functions. The mass spectrum of 147 has a molecular ion peak at m/z 316 and a base peak at m/z 283 corresponding to M⁺- CH₃, H₂O. The remaining peaks are of low intensity; however diagnostic peaks are observed at m/z 215 and 189 (Scheme 10). In the $^1$H NMR spectrum, signals are present for 3 tertiary methyl groups (δ 0.96, 1.05 and 1.20), an isopropyl group (δ 1.20, d, CH₃; δ 1.21, d, CH₃ and δ 3.28, septet, benzylic methine), and a methoxyl group at δ 3.72. Also observed are signals at δ2.76 (1H, ddd, H7α) and 3.02 (1H, br dd, H7β) indicating a benzylic methylene, δ 3.51 (1H, br t, W¹ = 7.0 Hz, CHO⁻OH) indicating an axially oriented hydroxyl functionality, and two aromatic signals at δ 7.04 (1H, s) and 7.05 (1H, s).
The characteristic signals in the $^{13}$C NMR spectrum of 147 are seen at $\delta$ 60.2 (OCH$_3$), 75.5 (CHOH), and 6 aromatic signals at $\delta$ 120.4, 123.7, 128.5, 137.9, 148.9 and 154.8. The APT spectrum confirms the presence of 6 methyl groups ($\delta$ 22.1, C10 CH$_3$; 23.9, 24.0, isopropyl methyl groups; 24.7, 25.9, C4 methyl groups and 60.2, OCH$_3$), a secondary alcohol ($\delta$ 75.5) and a tetrasubstituted benzene ring (the signals at $\delta$ 120.4 and 123.7 are inverted).

A 2D $^1$H-$^1$H COSY spectrum of 147 (Figure 9) shows coupling of the downfield proton at $\delta$ 3.51 with 2 other protons (3$\beta$/2, 3$\beta$/2') which show geminal coupling (2/2') and show cross peaks to 2 other protons (2'/1, 2'/1', 2/1, and 2/1'). The benzylic proton at C15 shows the expected coupling with a cross peak to the isopropyl methyl groups (15/16,17) while the benzylic protons at C7 show cross peaks to the protons at C6 (7$\beta$/6, 7$\beta$/6', 7$\alpha$/6 and 7$\alpha$/6'). Coupling of H5 with H6 and H6' is obscured by diagonal crowding.
Scheme 10 Mass spectral fragmentation for diterpene 147
Figure 9 COSY spectrum of 147
Figure 10 SINEPT spectrum of 147
A SINEPT experiment (Figure 10) was carried out on 147 to determine whether the methoxyl group is attached to C11 or C12. Irradiation of the signal at δ 3.28 (H15) in the $^1$H NMR spectrum was expected to enhance carbons 12 and 14 in the $^{13}$C NMR spectrum (enhancement of carbon through three bonds ($^{3}$J$_{C,H}$) via polarization transfer from hydrogen). Irradiation of the signal at δ 3.28 (H15), however, resulted in enhancement of signals in the $^{13}$C NMR spectrum at δ 123.7, 128.5, 137.9, 148.9 and 154.8. Since the experiment was set up to enhance the $^{3}$J$_{C,H}$ coupled carbons (ie. C12 and C14), the result was rather surprising. Nevertheless, the result indicates that attachment of the methoxyl group is at C12 since only one of the unsubstituted benzene carbons is enhanced (δ 123.7). Also, the quaternary carbon at δ 154.8 (the most deshielded carbon) is not observed in the $^{13}$C NMR spectrum, but is quite strongly enhanced in the SINEPT spectrum, placing the methoxyl group at C12.

A series of nOe difference experiments were carried out in order to confirm that the hydroxyl group is situated at C3 and to provide further evidence for the location of the methoxyl group at C12 (Figure 11). Irradiation of OCH$_3$ at δ 3.72 shows enhancement of the isopropyl methine proton at δ 3.28 while irradiation of the latter produced enhancement of the OCH$_3$ signal. This evidence supports the assignment of the OCH$_3$ group to C12. Irradiation of the signal at δ 3.51 (CHOH) produced enhancements of both the C4 methyl groups at δ 0.96 and 1.05 confirming the location of the hydroxyl group at C3α (axial).

The spectral evidence confirms the structure of 147 as 12-methoxyabieta-8,11,13-trien-3α-ol. Its isolation from the tissue cultures of *Tripterygium wilfordii* constitutes its first isolation from such an experiment.

Fractions J and K

Column chromatography of the combined fractions, J and K, by stepwise elution using: i) methylene chloride-ethyl acetate 19:1 and ii) methylene chloride-ethyl acetate 9:1
Figure 11 NOe difference spectra of 147
yielded 9 crude fractions. Further separation of fractions 1 and 2 by preparative TLC (chloroform-methanol 98:2 and toluene-ethyl acetate-chloroform-formic acid 35:15:16:1) yielded the quinone methide 148 (15.30 mg) and the friedelane methyl ester, 149 (13.62 mg). Fraction 3 yielded further quantities of 148 (14.00 mg) and the quinone methide, 150 (13.04 mg), by preparative TLC (toluene-ethyl acetate-chloroform-formic acid 35:15:16:1). Further quantities of 150 (15.55 mg) were obtained by preparative TLC of fractions 4 and 5 (chloroform-methanol 98:2). Column chromatography of fractions 6 and 7 (methylene chloride-ethyl acetate 5:1) yielded the triterpenes, 151 (50.75 mg) and 152 (28.48 mg) while fractions 8 and 9 (column chromatography, methylene chloride-ethyl acetate 5:1) yielded polpunonic acid (55) (247.13 mg).

The quinone methide, 148, was isolated as optically active ([α]_D^{22} -82.6) dark orange crystals (mp 140-142°C) with a molecular formula of C_{28}H_{36}O_{3}. The UV spectrum gave λ_{max} 225 (ε 5.13x10^3), 256 (ε 8.20x10^3) and 424 nm (ε 8.72x10^3) and the IR spectrum shows OH (3530-3365 cm\(^{-1}\)), carbonyl (1701 and 1645 cm\(^{-1}\)) and alkene (1600 cm\(^{-1}\)) absorption bands. The mass spectrum of 148 shows a strong molecular ion peak at m/z 420. The principal fragment ions occur at m/z 406, 405, 267, 253, 241, 227, 202 and 201, consistent with fragmentation at rings C and D of quinone methide triterpenes (Scheme 11). The high resolution mass spectrum confirms the molecular formulae of all the fragments proposed in Scheme 11 providing further evidence for the ABC ring structure of 148. A peak at m/z 436 is present in the spectrum and is probably due to the presence of a similar compound with an extra hydroxyl group, as an impurity. Its \(^1\)H NMR spectrum reveals the presence of 6 methyl groups at δ 0.99 (3H, s), 1.00 (3H, d), 1.02 (3H, s), 1.35 (3H, s), 1.52 (3H, s) and 2.23 (3H, s). The C22 protons are evident as doublets at δ 1.87 (H22β, J = 14.0 Hz) and 2.92 (H22α, J = 14.0 Hz). The C20α proton is manifest as a multiplet at δ 2.50 and H7, H1 and H6 appear at δ 6.39 (1H, d, J = 8.0 Hz), 6.56 (1H, d, J = 1.0 Hz) and 7.05 (1H, dd, 1.0, 8.0 Hz) respectively. The C3 hydroxyl signal is evident at δ 6.99. Ten downfield signals are evident in the \(^13\)C NMR spectrum at
δ 117.2, 118.0, 119.8, 127.6, 133.9, 146.0, 164.7, 168.7, 178.2 and 213.8 corresponding to the AB ring carbon atoms (C1 to C8 and C10) and a carbonyl group at C21. The APT spectrum shows eight inverted signals between δ 10.0 and 44.0 corresponding to 6 methyl groups and 2 aliphatic CH groups. Inverted signals at δ 118.0, 119.8 and 133.9 correspond to the alkene carbon atoms at C7, C1 and C6.

In the 1H-1H COSY spectrum (Figure 12) the signal at δ 2.92 (H22α) shows 2 cross peaks. One cross peak is a result of long range W coupling to a methyl group (C17 CH₃) and the other is due to geminal coupling to H22β (22α/22β). Coupling of the protons attached to C30, C20, C19, and C18 can be traced by the cross peaks at δ 2.50 (20α/30, 20α/19β and 20α/19α), 2.17 (19α/18β, 19α/19β) and 1.75 (19β/18β) which can only occur if the ketone is located at C21. The strong cross peak observed at δ 2.50 for 20α/19β suggests an axial-axial coupling between H20 and H19β (from model studies) which supports the assignment of H20 as α. The structure and relative stereochemistry of 148 were confirmed by a single crystal X-ray analysis 76 and is shown to be identical with tingenone.80,81 The computer-generated X-ray structure is given in Figure 13 and shows rings C, D and E in the expected chair conformation. Also evident is the incorporation of ethyl acetate in the crystal lattice.

The isolation of tingenone (148) constitutes its first isolation from tissue cultures of Tripterygium wilfordii. Previous isolation work from TRP-4a tissue cultures resulted in the isolation of a similar compound, 125, which was found to be not identical with tingenone by TLC.57 Tingenone has been found to demonstrate cytostatic activity on HeLa cells 82 and spectroscopic evidence has been provided for its interaction with DNA.83 Cytotoxicity against KB cells (of the human nasopharynx) has also been demonstrated.84

The friedelane methyl ester, 149, was isolated as optically active ([α]D +101.4) yellow crystals (mp 150-151°C) with a molecular formula of C₃₀H₄₆O₅. The IR spectrum shows absorptions for a hydroxyl group (3460 cm⁻¹), carbonyl groups (1725, sh, 1710, and 1700, sh) and a methoxyl group of an ester (1205 cm⁻¹).
Scheme 11 Proposed mass spectral fragmentation for 148
Figure 12 COSY spectrum of 148
Figure 13 Computer generated X-ray structure of 148
The mass spectrum of 149 gives a strong molecular ion peak at m/z 486 (base peak) and a strong peak at m/z 426 (M+ - HCO2CH3) which suggests that the methyl ester is at an angular position (C5, C9, C12, C13, C14 or C17) with C5 or C17 being the most likely locations considering the ease with which HCO2CH3 is eliminated. Support for the location of one ketone and the hydroxyl group in ring E is given by a peak at m/z 399 (Scheme 12).75 A peak at m/z 317 (which further loses HCO2CH3 to give m/z 257) locates the methyl ester and the remaining ketone in the ABC fragment. The molecular formulae given by the high resolution mass spectrum support the fragmentation pattern proposed in Scheme 12. The 1H NMR spectrum indicates seven methyl groups (5 singlets and 2 doublets) at δ 0.80 (3H, s), 0.84 (3H, s), 0.91 (3H, d), 0.94 (3H, s), 1.07 (3H, d), 1.36 (3H, s) and 3.64 (3H, s, CO2CH3). A signal at δ 3.65 disappeared on addition of D2O, and resulted in the signal at δ 4.61 (1H, d) collapsing to a broad singlet (H22), thus suggesting the presence of a secondary hydroxyl group. The downfield shift of the latter signal indicates that this functionality is adjacent to an electron-withdrawing group (in this case, carbonyl). A signal at δ 2.77 (1H, m) is suggestive of a proton adjacent to a carbonyl group. Comparison with the 1H NMR spectrum of tingenone (148) suggests a C20α proton adjacent to a C21 ketone functionality. Signals in the region δ 2.00 to 2.65 (3H) point towards the presence of a second ketone functionality in the molecule, presumably at C3 (from biosynthetic considerations).

Confirmation of the structure of 149 was achieved by a single X-ray analysis.76 The absolute stereochemistry (by anomalous dispersion) is that shown in the computer-generated X-ray structure (Figure 14). The methyl ester is established at C5 and all the rings are in the expected chair conformation.

The friedelane methyl ester, 149, is a novel triterpene exhibiting an unusual oxidation at C5. Its isolation from TRP-4a cultures and its structural similarity to the quinone methide triterpenes, 148 and 150, suggest a common biosynthetic pathway (vide infra).
Scheme 12 Proposed mass spectral fragmentation for 149
Figure 14 Computer generated X-ray structure of 149 (the absolute stereochemistry is shown)
The quinone methide, 150, was isolated as optically active ([a]$_D^{22}$ -484.6) dark orange crystals (mp 220-224°C) with a molecular formula of C$_{28}$H$_{36}$O$_4$. Its UV spectrum shows absorptions at $\lambda_{\text{max}}$ 233 (e 4.68x10$^3$), 254 (e 6.68x10$^3$), 264 (e 5.35x10$^3$) and 426 nm (e 1.05x10$^4$). Absorption bands are observed in the IR spectrum for hydroxyl (3450 cm$^{-1}$), carbonyl (1701 and 1648 cm$^{-1}$), and alkene (1598 cm$^{-1}$) groups. The mass spectral fragmentation pattern for 150 is very similar to that of tingenone (148), showing fragment ions at m/z 253, 241, 227, 202 (base peak) and 201 (see Scheme 11). Compound 150, does, however, show a strong molecular ion peak at m/z 436 and fragment ion peaks at m/z 423 and 422. Six methyl group signals are observed in the $^1$H NMR spectrum at $\delta$ 0.88 (3H, s), 0.99 (3H, s), 1.08 (3H, d), 1.37 (3H, s), 1.52 (3H, s) and 2.23 (3H, s, C4 CH$_3$). The proton at C20 is evident as a multiplet at $\delta$ 2.67 and H22 is seen as a singlet at $\delta$ 4.56. Exchangeable protons appear at $\delta$ 3.67 (C22 OH) and 7.00 (C3 OH). Signals characteristic of quinone methide triterpenes appear at $\delta$ 6.41 (H7), 6.56 (H1) and 7.06 (H6). The $^{13}$C NMR spectrum reveals a hydroxyl-bearing carbon at $\delta$ 76.3 and 10 sp$^2$ carbons at $\delta$ 117.3, 118.0, 119.9, 127.6, 134.0, 146.0, 164.7, 168.7, 178.2 and 213.3 corresponding to C1 to C8, C10 and a ketone group at C21. The APT spectrum reveals 8 inverted signals corresponding to 6 methyl groups and 2 methine protons, an inverted signal due to CHOH and 3 inverted sp$^2$ signals at $\delta$ 118.0, 119.9 and 134.0 corresponding to C7, C1 and C6.

The $^1$H-$^1$H COSY spectrum for 150 (Figure 15) shows similar cross peaks to those of 148 at $\delta$ 2.67 (20α/30, 20α/19β and 20α/19α), 2.19 (19α/19β, 19α/18β) and 1.78 (18β/19β) indicating a similar structure for rings D and E. A cross peak is also observed at $\delta$ 4.56 (22α/C22 OH) indicating coupling of H22α with the hydroxyl proton at C22.

The structure and relative stereochemistry of 150 were confirmed as 22β-hydroxytingenone by a single crystal X-ray analysis (Figure 16).$^{76}$ Rings C, D and E are shown to be in the expected chair conformations. The ketone functionality is confirmed at C21 and the hydroxyl group is shown to be at C22β.
Figure 15 COSY spectrum of 150
Figure 16 Computer generated X-ray structure of 150
The isolation of 22β-hydroxytingenone (150) constitutes its first isolation from tissue cultures of *Tripterygium wilfordii*. The tingenones 148 and 150 have been isolated previously from several plants of the Celastraceae family including *Euonymus tingens* and various *Maytenus* species.80-86

The dihydroxyoleanene, 151, was isolated as optically active ([α]D22 +115.9) colourless prisms (mp 241-242°C) with a molecular formula of C30H50O2. Its IR spectrum shows a strong hydroxyl band at 3294 cm⁻¹. The mass spectrum of 151 shows a molecular ion peak at m/z 442 and a base peak at m/z 234 corresponding to the RDA fragmentation of an oleanene skeleton. Peaks are also observed at m/z 427 (M⁺- CH₃), 411(M⁺- CH₂OH), 219 (RDA - CH₃) and 203 (RDA - CH₂OH). The mass spectral pattern places the hydroxymethyl group in the DE fragment of the molecule at C14, C17 or C20. Seven methyl group singlets appear in the ¹H NMR spectrum at δ 0.80, 0.85, 0.91, 0.94, 0.97, 1.00 and 1.14. An olefinic proton signal is observed at δ 5.21 and a one proton multiplet is observed at δ 3.22 (dd, CHOH, J = 3.5, 11.0 Hz) indicating a C3β hydroxyl group. A two proton doublet at δ 3.27 collapsed to a singlet on addition of D₂O suggesting the presence of a primary hydroxyl group (D₂O addition did not, however, reveal the location of the hydroxyl protons, probably due to masking in the aliphatic region of the spectrum). A single crystal X-ray analysis 76 provided the relative stereochemistry of 151 and location of the hydroxymethyl group at C20α. The computer-generated X-ray structure is given in Figure 17.

The isolation of 151 constitutes its first isolation from tissue cultures of *Tripterygium wilfordii*. The same compound is reported to be isolated from *Celastrus paniculatus* 87 and given the trivial name paniculatadiol (no spectral data, however, were presented).

Shibata et al. have found, from extensive studies on structure-activity relationships, that the hydroxymethyl group is important in the pharmacological effects of oleanene-type triterpenoids.88 11-Deoxoglycyrrhetol (153), derived from glycyrrhetinic
Figure 17 Computer generated X-ray structure of 151
acid (154), was shown to inhibit stress-induced peptic ulcer and various types of allergy. A variety of saikosaponins and polygalacins (triterpenoid saponins containing a hydroxymethyl group in the triterpene skeleton) were shown to be effective antiinflammatory agents. Extensive research has shown that hemiphthalate derivatives of deoxoglycyrrhetol (153) and the diene compounds, 155 and 156, possess strong antitumour and analgesic activity and are useful for the treatment of skin inflammations.\textsuperscript{89-94} The finding that the hydroxymethyl functionality is important in the pharmacological
activities of triterpenoids, suggests that the dihydroxy compound 151 might also possess some interesting pharmacological activities.

The dihydroxy triterpene, 152, was isolated as optically active ([α]_D^2^ +55.3) colourless prisms (mp 230-234°C) with a molecular formula of C_{30}H_{50}O_{2}. Two hydroxyl bands are evident in the IR spectrum at 3675 and 3605 cm^{-1}. The molecular ion peak of 152 (m/z 442) in the mass spectrum is very weak and a base peak at m/z 424 is observed due to M^+ - H_2O. Other peaks are observed at m/z 409 (M^+ - CH_3, H_2O) and 391 (M^+ - CH_3, 2H_2O). A weak RDA fragment ion is observed at m/z 234 with other fragmentations predominating due to the presence of hydroxyl at C11. The ^1H NMR shows eight methyl group singlets at δ 0.83, 0.86, 0.91 (6H, s), 1.03 (6H, s), 1.08 and 1.23. Two methine proton signals are also observed at δ 3.26 (dd, J = 5.0, 10.0 Hz) and 4.21 (dd, J = 2.5, 7.5 Hz) corresponding to CHOH at C3α and C11β respectively. The H12 olefinic signal at δ 5.27 is observed as a doublet with J = 2.5 Hz indicating coupling to H11β. The ^13C NMR spectrum shows 2 COH signals at δ 67.6 and 78.7 corresponding to C3 and C11 respectively, and 2 sp^2 carbons at δ 125.4 and 149.5 corresponding to C12 and C13 respectively. The APT spectrum shows 8 inverted methyl group signals at δ 15.5, 16.9, 18.1, 19.8, 23.6, 28.1, 28.5 and 33.3, and inverted signals for CHOH at δ 67.6 and 78.7 and for C12 at δ 125.4. Cross peaks in the ^1H-^1H COSY spectrum (Figure 18) are evident at δ 5.27 (12/11β) and 4.21 (11β/9α, where 9α appears at δ 1.55), showing the AMX system at C12, C11, C9. The H3α proton at δ 3.26 shows a cross peak to a signal at δ 1.66 (3α/2,2') which in turn shows cross peaks to signals at δ 2.00 (2,2'/1) and 1.22 (2,2'/1'). Geminal coupling is observed between H1 and H1' by a cross peak at δ 2.00.

Confirmation of the structure and relative stereochemistry of 152 was provided by a single crystal X-ray analysis. The computer-generated X-ray structure (Figure 19) shows rings A, B and E in the expected chair conformations. The C11 hydroxyl group is confirmed as α-oriented (equatorial) and is seen as hydrogen-bonded to one molecule of ethanol (from the recrystallization solvent) which is incorporated into the crystal lattice.
Figure 18 COSY spectrum of 152
Figure 19  Computer generated X-ray structure of 152
The isolation of 11α-hydroxy-β-amyrin (152) constitutes its first isolation from tissue cultures of *Tripterygium wilfordii*. A previous isolation of 11α-hydroxy-β-amyrin was carried out from the gummy secretion of *Salvia glutinosa*.95

Polpunonic acid (55) was isolated as optically active ([α]$_D^{22}$ -60.0) off-white needles (mp 246-248°C) with a molecular formula of C$_{30}$H$_{48}$O$_3$. Its IR spectrum shows carbonyl bands at 1740 and 1702 cm$^{-1}$. The $^1$H NMR and mass spectra are identical to those in the literature.57,96 Polpunonic acid has been isolated previously from tissue cultures of *Tripterygium wilfordii*57 and from the roots of *Plenckia polpunea*.96 Antibacterial and antimitotic activity have been demonstrated for this compound.

2.3.3 Isolation of metabolites from the spent medium extract of TRP-4a

The spent medium extract (3.06 g) was subjected to VLC by stepwise elution using: i) benzene; ii) benzene-acetone 5:1; iii) benzene-acetone 4:1; iv) benzene-acetone 3:1; v) benzene-acetone 2:1; vi) benzene-acetone 1:1 and vii) ethyl acetate to give 22 crude fractions. Oleanolic acid (127, 59.66 mg), the diterpene hydroxy acid, 160 (134.63 mg) and triptolide (2, 179.09 mg) were isolated from the first seven crude fractions by preparative TLC using toluene-ethyl acetate-chloroform-formic acid 35:15:16:1. Preparative TLC of fractions 8-13 using the same eluent yielded the triterpenes 137 (13.22 mg), 138 (21.72 mg), 139 (8.73 mg) and 51 (104.30 mg), and tripdiolide (1, 199.77 mg). Preparative TLC separation of fractions 14-16 (toluene-ethyl acetate-chloroform-formic acid 35:15:16:1) and 17 and 18 (benzene-methanol-acetic acid 90:5:5) yielded further quantities of triterpene 51. Fractions 19-22 were complex mixtures of polar compounds. Attempted separation by preparative TLC was unsuccessful.
The hydroxy acid, 160, was isolated as optically active ([α]D22 +143.9) colourless needles (mp 141-145°C) with a molecular formula of C20H26O3. Its IR spectrum shows absorption bands for hydroxyl (3600 cm\(^{-1}\)), carboxylic acid (2580 cm\(^{-1}\)), carbonyl (1677 cm\(^{-1}\)) and alkene (1618 cm\(^{-1}\)) functions. The C10 methyl group signal appears in the \(^1\)H NMR spectrum at δ 1.06 (3H, s) and isopropyl methyl groups appear at δ 1.58 (6H, s). The C4 methyl group signal appears downfield at δ 2.14 (3H, br s). A two proton multiplet at δ 3.02 corresponds to the benzylic protons at C7 and the aromatic protons are evident as a multiplet signal between δ 7.25 and 7.33. A molecular ion peak is observed in the mass spectrum at m/z 314 with fragment ion peaks at m/z 299 (M\(^+\)-CH\(_3\)), 296 (M\(^+\)-H\(_2\)O), 281 (M\(^+\)-CH\(_3\), H\(_2\)O), 263 (M\(^+\)-CH\(_3\), 2H\(_2\)O), 253 (M\(^+\)-HCO\(_2\)H, CH\(_3\)) and 235 (M\(^+\)-HCO\(_2\)H, CH\(_3\), H\(_2\)O).

The hydroxy acid, 160, has been isolated previously from the TRP-4a cultures as the methyl ester, 124.\(^{57}\) The structure of the methyl ester was proven by synthesis according to Scheme 8.

Triptolide (2) was isolated as colourless needles (mp 230-234°C) and tripdiolide (1) was isolated as colourless plates (mp 224-226°C). Their TLC, IR, mp, \(^1\)H NMR and mass spectra were identical with authentic samples (isolated previously by Kutney et al.).\(^{57}\)
In summary, the extraction of the TRP-4a suspension cultures and separation of the extracts led to the isolation of eighteen compounds, mainly triterpenes and also some diterpenes. Twelve of the compounds have not been isolated previously from the TRP-4a cultures, 5 of which are new compounds (137, 138, 139, 147 and 149). The diterpene triepoxides, 1 and 2, polpunonic acid (55), the hydroxy acid, 160 (as the methyl ester, 124), β-sitosterol (128) and oleanolic acid (127) have been isolated previously from the TRP-4a cultures. The chromatographic separations of the metabolites from the cell and spent medium extracts are summarized in Figures 20 and 21 respectively and the yields (from 78 L of tissue culture) are summarized in Table 8.

2.4 Biological activities of the isolated metabolites

Based on preliminary screening of crude fractions and available quantities of materials, the purified triterpenes 137, 138, 139 and 51, oleanolic acid (127), triptolide (2) and tripdiolide (1) were submitted for antifertility testing. The results of in vivo antifertility testing showed that triptolide and tripdiolide are active in suppressing fertility in male SD rats. Triptolide was shown to be 7 times more potent than tripdiolide. The triterpenes 137, 138, 139 and 51 were found to be ineffective at the largest possible dose levels. Immunological assays were carried out on 138, 139, 51, oleanolic acid (127) and the diterpenes 160, 1 and 2. Preliminary screening of the crude fractions had shown that the lymphocyte proliferation test (by 3H-thymidine incorporation into lymphocyte DNA) was the most sensitive parameter for these samples. Inhibition of lymphocyte proliferation was thus demonstrated for triptolide (2) and tripdiolide (1) with

1 Antifertility assays were carried out at the Jiangsu Family Planning Institute, Nanjing, China.
2 Immunological assays were carried out at the Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China.
triptolide inhibiting lymphocyte DNA synthesis at a concentration of 0.32 ng/mL (Table 9). The hydroxy acid diterpene, 160, showed activity at the 200 ng/mL level while the triterpenes were inactive.

Figure 20 Chromatographic separation of the cell extract
Figure 21 Chromatographic separation of the spent medium extract
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Table 8 Yields of the metabolites isolated from 78 L of TRP-4a suspension culture
Table 9  
Inhibition of lymphocyte proliferation by TRP-4a metabolites. Con A induced $^3$H-thymidine incorporation test *in vitro*.

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1 Samples were added directly to mice spleen cells in a culture medium with mitogen Con A (Sigma, 5 μg/mL). $^3$H-Thymidine was added after 72 hours incubation at 37°C in a 5% CO₂ incubator. Radioactivity incorporated into cell DNA was measured by liquid scintillation counting.
Scheme 13 Biogenetic relationship between oleanene- and friedelane-type triterpenes
Scheme 14 Proposed biogenetic relationship between polpunonic acid (55) and the quinone methides
Scheme 15 Proposed biogenesis of the quinone methides from polpunonic acid (55) via C24 oxidation and elimination
From the results of the antifertility testing, triptolide (2) and triptolide (1) look promising as potential male contraceptive agents, although the strong immunosuppressive activities of these compounds indicate a possible limitation to their use as antifertility agents. Research is ongoing at the Institutes in China at the present time, to determine whether the active compounds possess immunosuppressive effects at the dose levels required for antifertility. Although the triterpenes were found to be ineffective, thus far, as antispermatic agents, research is still continuing and further antifertility tests are planned when sufficient quantities of the appropriate metabolites become available.

2.5 Biogenetic relationship of the triterpenes

The oleanene and friedelane skeletons have been related to a common pentacyclic precursor (derived from 2,3-epoxysqualene) via a series of hydride and methyl shifts (Scheme 13). Further enzymatic oxidations of these basic skeletons leads to the variety of oleanene- and friedelane-type triterpenes present in the TRP-4a tissue cultures.

The quinone methides celastrol (46), tingenone (148) and 22β-hydroxytingenone (150) exhibit a friedelane-type skeleton. A common biogenesis between the friedelanes and the quinone methides was first suggested by Marini-Bettollo et al. in which polpunonic acid (55) was postulated to be the precursor of pristimerin (165). Kutney et al. later proposed biogenetic relationships between polpunonic acid (55), salaspermic acid (53) and the quinone methides (Scheme 14).

The isolation of the novel friedelane methyl ester, 149, provides further support for the biogenetic relationship between the friedelanes and the quinone methides. Given that methyl group removal proceeds via oxidation by monooxygenase enzymes followed by elimination of CO$_2$ or formic acid, possible biosynthetic pathways from polpunonic acid
to the quinone methides might proceed according to Scheme 15. Direct evidence in terms of biosynthetic experiments is lacking.
The previously discussed antileukaemic activity of the diterpene triepoxides raised considerable interest in their production, not only by synthesis, but also by production from plant tissue cultures. In this regard, interest in the biosynthetic pathway to these compounds was stimulated and biotransformation studies using "late" abietane-type synthetic precursors was pursued. The renewed interest in the diterpene triepoxides based on the recent discovery of their antifertility activity has promoted also the elucidation of their biosynthetic pathway. It is hoped that an understanding of the biosynthetic pathway to the diterpene triepoxides will lead to increased production of the diterpene triepoxides from TRP-4a tissue cultures by addition of the appropriate synthetic precursors. In this way, it is hoped that the length of time required for the production of the diterpene triepoxides can be reduced considerably with a view to their commercial production in the future.

The isolation of dehydroabietic acid (80) and the hydroxy ester, 124, as co-occurring metabolites from the TRP-4a tissue culture line, led to proposals of the biosynthetic pathway to tripdiolide (1).\(^{99}\) A biosynthetic pathway from geranylgeraniol through dehydroabietic acid (80) and the acid, 135, was proposed (Scheme 16). Alternatively, methyl group transfer was proposed from dehydroabietane (168) followed by oxidation and butenolide formation, prior to oxidation of ring C to give tripdiolide (1), (Scheme 17).

Previously, chemically related precursors possessing an abietane-type structure have been synthesised in an effort to investigate the biosynthetic pathway subsequent to dehydroabietic acid (80).\(^{57,100}\) Preliminary biotransformation studies of dehydroabietic acid (80) and the hydroxy ester, 124, in radioactive form,\(^{99}\) as well as of the synthetic precursors, 135 and 169-174, were carried out using the TRP-4a cultures.\(^{101}\) The most
Scheme 16 Proposed biosynthetic pathway to tripdiolide (1) via dehydroabietic acid (80)
promising results were obtained using the allylic alcohol, 171, which showed considerable utilization by the cells. In light of these preliminary results, it was decided to investigate further the biotransformation of 171, and work was undertaken to synthesise the radioactive form of 171 in order that the biotransformation might be examined in greater detail.

Scheme 17 Proposed biosynthesis of tripdiolide (1) via 4→3 methyl transfer
2.6 Synthesis of the allylic alcohol 171

The allylic alcohol was synthesised according to Scheme 18. Dehydroabietic acid (80) was converted to the ketone, 180, using Huffman’s procedure.\textsuperscript{102, 103} Decarboxylation of 80 with lead tetraacetate led to the olefin mixture, 175, which on hydroboration-oxidation yielded the mixture of alcohols, 176. Separation of the alcohol
mixture was carried out by preparative HPLC to give the 3β alcohol, 177, the 3α alcohol, 178 and the primary alcohol, 179 in the ratio 1:3:3, respectively. The alcohol, 177, proved to be unstable and oxidation with a variety of chromium oxidizing agents led to intractable mixtures. Oxidation of 178 with chromium trioxide-pyridine complex, on the other hand, proceeded cleanly to the ketone, 180. The yield of the ketone from dehydroabietic acid (80) was 10%. The stereochemistry of 180 at C4 was established by a $^1$H NMR decoupling experiment. Irradiation of the doublet at δ 1.21 corresponding to the C4 methyl group led to collapse of the multiplet at δ 2.57 (corresponding to the proton attached to C4) to a doublet with a residual coupling of 6 Hz. The magnitude of the coupling places the C4 hydrogen in the α position giving an equatorial-axial coupling with the hydrogen at C5.

Conversion of the ketone, 180, to the allylic alcohol, 171, was executed via a Shapiro reaction $^{104, 105}$ and coupling with paraformaldehyde. Condensation of 180 with toluenesulphonylhydrazide (tosylhydrazide) using boron trifluoride etherate over a period of 40 hours gave the tosylhydrazone, 181, in almost quantitative yield. Irradiation of the C4 methyl signal at δ 1.09 in the $^1$H NMR spectrum resulted in the collapse of the multiplet at δ 2.23, corresponding to the C4 hydrogen, to a doublet with a residual coupling of 11 Hz. The stereochemistry of the C4 hydrogen must therefore be β giving an axial-axial coupling with the C5 hydrogen. Shorter reaction times led to mixtures of the two C4 isomers as evidenced by the $^1$H NMR spectrum. Addition of n-butyllithium to the tosylhydrazone, 181, followed by trapping of the resulting anion with paraformaldehyde gave the allylic alcohol, 171, in 55% yield.

Synthesis of the radioactive allylic alcohol, 182, was carried out in the same way as for the inactive alcohol, 171, except that 500 μCi ($1.11\times10^9$ DPM) of $^{14}$C paraformaldehyde were added and the reaction mixture was allowed to stir for 1 hour before the addition of the inactive paraformaldehyde. A low incorporation of the radiolabel into the final product was obtained (0.4%, $4.68\times10^6$ DPM). However, the level of incorporation was considered to be sufficiently high to proceed with the biotransformation studies using the TRP-4a tissue cultures. Sufficient quantities of both the radioactive and
inactive allylic alcohols were thus available to conduct concurrently experiments with radioactive and inactive substrates.

Scheme 18 Synthesis of the allylic alcohol, 171

a) PbOAc₄, pyridine, PhH, Δ; b) LAH, Et₂O, BF₃.OEt₂, THF, NaOH, H₂O₂; c) Chromatography; d) CrO₃.2pyr.; e) TsNHNH₂, BF₃.OEt₂, PhH; f) n-BuLi, (CH₂O)n, TMEDA.
2.7 Biotransformation of the allylic alcohol, 171 and the radioactive allylic alcohol, 182

Preliminary experiments carried out earlier with the inactive allylic alcohol, 171, showed that the optimum conditions for biotransformation were incubation of the alcohol with the TRP-4a cultures for 120 hours. These conditions were chosen to carry out the large scale biotransformation experiments in which radioactive and inactive precursor were incubated separately and simultaneously with the cultures.

The cultures were grown, as previously, in PRD2Co100 growth medium and resuspended in the MSNA0.8K0.5 production medium, and incubated with the precursor until the end of the growth phase (26 days). Five hundred milligrams of the inactive allylic alcohol, 171, was added to 5 L of the suspension culture and 100 mg (4.59×10⁶ DPM) of the radioactive allylic alcohol, 182, was added to 1 L of the suspension culture and the cultures were incubated for a further 120 hours.

Harvesting and extraction of the cultures from the experiments involving radioactive and inactive precursors was carried out as previously described in Part A. The cells were separated from the spent medium by filtration and were extracted separately. The cells were extracted according to Figure 3 except that the ethyl acetate and the methanol extracts were combined. The spent medium was extracted according to Figure
4 except that a methanol extraction was not performed. Extraction of the culture in the radioactive experiment yielded 3.68 g (3.70×10^6 DPM) of extract from the cells and 174.10 mg (7.35×10^5 DPM) of extract from the spent medium, constituting a 97% (4.44×10^6 DPM) recovery of the radioactivity from the radioactive culture. Extraction of the culture in the inactive experiment yielded 5.48 g of cell extract and 281.30 mg of spent medium extract.

<table>
<thead>
<tr>
<th>Culture volume (L)</th>
<th>Amt. precursor added (mg)</th>
<th>Incubation time (hrs)</th>
<th>Wt (g)</th>
<th>DPM (×10^6)</th>
<th>Wt (g)</th>
<th>DPM (×10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radioactive</td>
<td>1.0</td>
<td>100</td>
<td>120</td>
<td>3.68</td>
<td>3.70</td>
<td>(83.4%)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inactive</td>
<td>5.0</td>
<td>500</td>
<td>120</td>
<td>5.48</td>
<td>-</td>
<td>0.28</td>
</tr>
<tr>
<td>experiment</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 10 Weights and activities of the extracts from the TRP-4a cultures incubated with radioactive and inactive precursors, 182 and 171.

Radioactivity profiles of the radioactive extracts were run on analytical TLC plates (20×20 cm) in order to determine the number of metabolites present in the extracts arising from biotransformation of the precursor. A radioactivity profile was run on a sample of the radioactive spent medium extract (5.86 mg, 3.42×10^4 DPM) using hexane-ethyl acetate 9:1 as the eluant. The bands of silica gel were removed from the TLC plate according to the location of the metabolites (as observed by visualization with anisaldehyde-H_2SO_4) and counted (Table 11 and Figure 22). A second radioactivity profile was run on the spent medium extract (5.30 mg, 3.09×10^4 DPM) using chloroform-
methanol-acetic acid 50:5:1 as the eluant in order to determine if any radioactivity was incorporated into the more polar metabolites (Table 12 and Figure 23).

![Chemical structures](image)

<table>
<thead>
<tr>
<th>Band No.</th>
<th>DPM (×10³)</th>
<th>% DPM ¹</th>
<th>%DPM total ²</th>
<th>Metabolite present</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.40</td>
<td>73.2</td>
<td>12.1</td>
<td>184</td>
</tr>
<tr>
<td>2</td>
<td>1.17</td>
<td>6.0</td>
<td>1.0</td>
<td>182</td>
</tr>
<tr>
<td>3</td>
<td>0.15</td>
<td>0.8</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3.76</td>
<td>19.1</td>
<td>3.2</td>
<td>183</td>
</tr>
<tr>
<td>5</td>
<td>0.04</td>
<td>0.2</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.14</td>
<td>0.7</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>

Table 11 Radioactivity profile data for the spent medium extract eluted with hexanes-ethyl acetate 9:1.

¹ % DPM for each band is calculated as a percentage of the radioactivity of the spent medium recovered from the TLC plate

² % DPM total for each band is calculated as a percentage of the total radioactivity recovered from the culture; e.g. 73.2 × 0.166 = 12.1 %DPM total. for band 1, where 0.166 is the percentage of spent medium activity of the total activity recovered from the culture (see Table 10).
The radioactivity profile of the spent medium extract, eluted with a non-polar solvent (hexanes-ethyl acetate 9:1), and TLC comparison with synthetic samples (see later for the syntheses) revealed the presence of the aldehyde, 183 (3.2% of the total recovered radioactivity), the starting material, 182 (1.0%) and the acid, 184 (12.1%) (Table 11, Figure 22). The radioactivity profile given in Table 12 and Figure 23 (chloroform-methanol-acetic acid 50:5:1) showed, however, some residual radioactivity in the more polar area (lower Rf bands) of the TLC plate. This could be due to irreversible binding of trace quantities of the radioactive compounds, 182, 183 and 184, or alternatively trace quantities of more polar metabolites which have not been accounted
for. Visualization of the TLC plate with anisaldehyde-H$_2$SO$_4$, however, did not reveal the presence of any metabolites in the polar region (bands 3 to 6 of Figure 23). One of the target compounds, tripdiolide (1) was observed in band 11 (Table 12, Figure 23). However, as is shown later, subsequent recrystallization revealed that 1 was not radioactive.

<table>
<thead>
<tr>
<th>Band No.</th>
<th>DPM ($\times 10^3$)</th>
<th>% DPM</th>
<th>%DPM total</th>
<th>Metabolite present</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.08</td>
<td>0.5</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.11</td>
<td>0.6</td>
<td>0.1</td>
<td></td>
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<td>3</td>
<td>0.35</td>
<td>2.0</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.18</td>
<td>1.1</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.30</td>
<td>1.7</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.47</td>
<td>2.7</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.38</td>
<td>2.2</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.21</td>
<td>1.2</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.26</td>
<td>1.5</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.66</td>
<td>3.8</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.23</td>
<td>1.3</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>4.24</td>
<td>24.3</td>
<td>4.0</td>
<td>184</td>
</tr>
<tr>
<td>13</td>
<td>4.59</td>
<td>26.3</td>
<td>4.4</td>
<td>184</td>
</tr>
<tr>
<td>14</td>
<td>0.85</td>
<td>4.9</td>
<td>0.8</td>
<td>182</td>
</tr>
<tr>
<td>15</td>
<td>4.53</td>
<td>26.0</td>
<td>4.3</td>
<td>183</td>
</tr>
</tbody>
</table>

Table 12 Radioactivity profile data for the spent medium extract eluted with chloroform-methanol-acetic acid 50:5:1
Before radioactivity profiles were carried out on the radioactive cell extract, a sample (1.39 g, $1.40 \times 10^6$ DPM) was filtered through silica gel in order to remove the large quantity of polar material resulting from the methanol extraction. Filtration through silica gel using ethyl acetate yielded the non-polar fraction (fraction 1, 60.25 mg, $7.79 \times 10^5$ DPM, 71.6% of the recovered activity) and rapid elution of the silica gel with methanol.
removed the polar material (fraction 2, 1.00 g, $3.09 \times 10^5$ DPM, 28.4% of the recovered activity). A considerable quantity of radioactivity remained in the polar fraction and therefore a radioactivity profile was carried out on both polar and non-polar fractions.

<table>
<thead>
<tr>
<th>Band No.</th>
<th>DPM ($\times 10^3$)</th>
<th>% DPM</th>
<th>%DPM total 1</th>
<th>Metabolite present</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13.02</td>
<td>32.2</td>
<td>19.2</td>
<td>184</td>
</tr>
<tr>
<td>2</td>
<td>5.22</td>
<td>12.9</td>
<td>7.7</td>
<td>182</td>
</tr>
<tr>
<td>3</td>
<td>1.34</td>
<td>3.3</td>
<td>2.0</td>
<td>183</td>
</tr>
<tr>
<td>4</td>
<td>18.96</td>
<td>46.9</td>
<td>28.0</td>
<td>183</td>
</tr>
<tr>
<td>5</td>
<td>0.35</td>
<td>0.9</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1.47</td>
<td>3.6</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.05</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.02</td>
<td>0.1</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.00</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.00</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

**Table 13** Radioactivity profile data of fraction 1 (cell extract) eluted with hexanes-ethyl acetate 9:1

1 The % DPM total is calculated from the % DPM for each band, eg $46.9 \times 0.72 \times 0.834 = 28.0$ where 0.72 is the percentage of fraction 1 activity of the cell extract and 0.834 is the percentage of cell extract activity of the total activity recovered from the culture.
A radioactivity profile was carried out on 4.09 mg (5.29×10^4 DPM) of fraction 1 using hexanes-ethyl acetate 9:1 in order to examine the non-polar metabolite content (Table 13 and Figure 24), and on 3.87 mg (4.99×10^4 DPM) using chloroform-methanol-acetic acid 95:5:1 in order to examine the more polar metabolite content (Table 14 and Figure 25). A radioactivity profile on fraction 2 (26.59 mg, 8.19×10^3 DPM) was run using chloroform-methanol-acetic acid 95:5:1 (Table 15 and Figure 26). No non-polar metabolites were observed in this fraction by TLC. A substantial quantity of the acid, 184, however, was observed.
The radioactivity profile of fraction 1 using hexanes-ethyl acetate 9:1 (Table 13, Figure 24) revealed the presence of the majority of the aldehyde, 183 (30.0% of the total recovered radioactivity), the allylic alcohol, 182 (7.7% of the total recovered radioactivity) and the acid, 184 (19.25% of the total recovered radioactivity). Elution with chloroform-methanol-acetic acid 95:5:1 (Table 14, Figure 25) showed that no other metabolites (more polar than the acid) were radioactive.

<table>
<thead>
<tr>
<th>Band No.</th>
<th>DPM (×10^3)</th>
<th>% DPM</th>
<th>%DPM total</th>
<th>Metabolite present</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.10</td>
<td>0.3</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.17</td>
<td>0.4</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.15</td>
<td>0.4</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.51</td>
<td>1.2</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>9.25</td>
<td>22.2</td>
<td>13.2</td>
<td>184</td>
</tr>
<tr>
<td>6</td>
<td>0.37</td>
<td>0.9</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>5.48</td>
<td>13.1</td>
<td>7.8</td>
<td>182</td>
</tr>
<tr>
<td>8</td>
<td>0.42</td>
<td>1.0</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1.22</td>
<td>2.9</td>
<td>1.7</td>
<td>183</td>
</tr>
<tr>
<td>10</td>
<td>24.10</td>
<td>57.7</td>
<td>34.5</td>
<td>183</td>
</tr>
</tbody>
</table>

Table 14 Radioactivity profile data of fraction 1 (cell extract) eluted with chloroform-methanol-acetic acid 95:5:1
The radioactivity profile of fraction 2 of the cell extract (chloroform-methanol-acetic acid 95:5:1, Table 15, Figure 26) showed that the residual radioactivity in this fraction was due to the presence of the acid, 184. No aldehyde or allylic alcohol were observed in this fraction. No tripdiolide (1) was observed in any of the cell extract fractions.
The results of the radioactivity profiles are summarized in Table 16. The same radioactive metabolites were found in the cell extract and the spent medium extract. The majority of the radioactive material was found in the cell extract while a small quantity of the metabolites was found in the spent medium extract, possibly due to cell rupture during the incubation period.

---

^e % DPM total is calculated from the % DPM for each band, eg 62.7 x 0.28 x 0.84 = 14.9 where 0.28 is the percentage of fraction 2 activity of the cell extract and 0.84 is the percentage of cell extract activity of the total activity recovered from the culture.
Since the radioactivity profiles revealed the presence of the same metabolites in the spent medium and the cell extracts, the remaining extracts were combined (3.80×10^6 DPM) prior to isolation of the radioactive metabolites. The combined extracts were
suspended in water and extracted into ethyl acetate to remove the bulk of the unwanted polar material. Solvent removal yielded a crude brown solid (309.70 mg, 3.42x10^6 DPM) which was subjected to column chromatography (chloroform-methanol-acetic acid 95:5:1). Seven crude fractions were obtained. Purification of fraction 2 by preparative TLC using hexanes-ethyl acetate 19:1 yielded the aldehyde, 183, as a colourless oil (19.86 mg, 8.13x10^5 DPM), while fractions 3 and 4 were purified by preparative TLC using chloroform-methanol-acetic acid 95:5:1 to give the starting alcohol, 182, (13.04 mg, 1.54x10^5 DPM) and a crude fraction of the acid, 184. The crude acid was combined with fractions 5 and 6 and preparative TLC using hexanes-ethanol-acetic acid 95:5:1 yielded purified acid, 184 (24.36 mg, 1.05x10^6 DPM). Tripdiolide (1, 2.02 mg, 6.92x10^3 DPM) was isolated from fraction 7 by preparative TLC using chloroform-methanol-acetic acid 95:5:1. Table 17 summarizes the quantities of the radioactive compounds obtained by chromatography and Figure 27 outlines the isolation procedure.

In order to confirm the structures of the radioactive metabolites, the corresponding metabolites were isolated from the parallel experiment involving the inactive precursor, 171. Partitioning of the cell extract (5.48 g) between ethyl acetate and water yielded a crude brown extract (1.09 g) which was subjected to column chromatography. Gradient elution involving increasingly polar solvents (hexanes to ethyl acetate) followed by rapid elution of the column with methanol, yielded 3 crude fractions. Fraction 1, containing the aldehyde, 185 and the alcohol, 171, was separated further by a series of preparative TLC plates to give 38.54 mg of the aldehyde as a colourless oil and 28.69 mg of the alcohol as a colourless oil. Fraction 2 contained the acid, 186, which was further purified by preparative TLC to give 123.23 mg of the acid as a white solid. Figure 28 outlines the isolation procedure for the inactive metabolites. The structures of the inactive metabolites were determined by comparison of the spectral data with authentic samples obtained via synthesis.
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>% DPM</th>
<th>Total % DPM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spent medium extract</td>
<td>Cell extract</td>
</tr>
<tr>
<td>183</td>
<td>3.2</td>
<td>30.0</td>
</tr>
<tr>
<td>182</td>
<td>1.0</td>
<td>7.7</td>
</tr>
<tr>
<td>184</td>
<td>12.1</td>
<td>40.0</td>
</tr>
</tbody>
</table>

Table 16 % DPM of the aldehyde, 183, allylic alcohol, 182, and acid, 184, in the spent medium and cell extracts, determined by the radioactivity profiles.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Wt. recovered (mg)</th>
<th>DPM ($\times 10^5$)</th>
<th>% DPM recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>183</td>
<td>19.86</td>
<td>8.13</td>
<td>21.4</td>
</tr>
<tr>
<td>182</td>
<td>13.04</td>
<td>1.54</td>
<td>4.1</td>
</tr>
<tr>
<td>184</td>
<td>24.36</td>
<td>10.50</td>
<td>27.6</td>
</tr>
<tr>
<td>Total recovery</td>
<td>57.26</td>
<td>20.17</td>
<td>53.1</td>
</tr>
</tbody>
</table>

Table 17 Quantities of the radioactive metabolites, 183, 182 and 184, isolated by chromatography.
Figure 27 Chromatographic separation of 182, 183, 184 and tripdiolide (1) from the radioactive extract
Crude extract, 1.09 g

Column chromatography, hexanes → EtOAc → MeOH

1

TLC, CHCl₃-MeOH-AcOH, 95:5:1

1

TLC, hexanes-ethyl acetate 9:1

Aldehyde, 185, 38.54 mg

TLC, CHCl₃-MeOH, 98:2

1

Acid, 185, 96.59 mg

2

Alcohol, 171, 24.85 mg

TLC, CHCl₃-MeOH-AcOH, 100:6:1

Alcohol, 171, 3.84 mg

TLC, CHCl₃-MeOH-AcOH, 95:5:1

1

Acid, 186, 26.64 mg

2

3

4

Figure 28 Chromatographic separation of 171, 185 and 186 from the inactive extract
The aldehyde, 185 and the acid, 186, were synthesised by oxidation of the allylic alcohol, 171 (Scheme 19). Oxidation using pyridinium chlorochromate yielded the aldehyde, 185, as a colourless oil (35.5%). In addition, oxidation of the allylic alcohol, 171, with Jones reagent (CrO₃-acetone-H₂SO₄) yielded the acid, 186, as a white solid (7.1%).

The specific activities of the radioactive metabolites, 183 and 184, were obtained by recrystallization until a constant specific activity was reached. The aldehyde, 183, was
converted to the 2,4-dinitrophenylhydrazone derivative, 187, in order to obtain a derivative suitable for crystallization. The specific activity of 183 is therefore based on the activity as determined from 187. Recrystallization of the 2,4-dinitrophenylhydrazone was carried out using ether-methylene chloride. The radioactive acid, 184, was first diluted with inactive material and recrystallized from hexanes. The tripdiolide fraction (2.02 mg, 6.92×10^3 DPM), was first diluted with 5.20 mg of an authentic sample of tripdiolide (1), obtained earlier in the TRP-4a cell experiments, and recrystallized from ethanol. The results are summarized in Table 18.

In summary, the biotransformation of the allylic alcohol, 171 (and 182) led only to relatively uninteresting oxidations of the alcohol functionality. Recrystallization of the tripdiolide fraction confirmed that no incorporation of 182 into tripdiolide (1) had taken place. The specific activities of the acid, 184 and the hydrazone, 187, as shown in Table 18 (5.88 and 5.85 mCi mol⁻¹ respectively) are consistent with that of the starting material, 182 (5.88 mCi mol⁻¹), indicating that no endogenous acid or aldehyde were present in the cultures. That is, the TRP-4a cell line does not produce 183 and 184 unless the precursor, 182 is added.
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>184</th>
<th>187</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Wt. (mg)</td>
<td>50.61</td>
<td>32.02</td>
<td>7.22</td>
</tr>
<tr>
<td>DPM (×10^5)</td>
<td>7.24</td>
<td>7.62</td>
<td>0.07</td>
</tr>
<tr>
<td>DPM mg⁻¹ (×10^4)</td>
<td>0.14</td>
<td>2.38</td>
<td>0.10</td>
</tr>
<tr>
<td>1st Wt. (mg)</td>
<td>20.14</td>
<td>20.99</td>
<td>3.43</td>
</tr>
<tr>
<td>Crystallisation DPM (×10^5)</td>
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<td>5.88</td>
<td>0.00</td>
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<tr>
<td>DPM mg⁻¹ (×10^4)</td>
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<td>2.80</td>
<td>0.00</td>
</tr>
<tr>
<td>2nd Wt. (mg)</td>
<td>16.24</td>
<td>13.91</td>
<td></td>
</tr>
<tr>
<td>Crystallisation DPM (×10^5)</td>
<td>2.37</td>
<td>3.91</td>
<td></td>
</tr>
<tr>
<td>DPM mg⁻¹ (×10^4)</td>
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<td>2.81</td>
<td></td>
</tr>
<tr>
<td>3rd Wt. (mg)</td>
<td>1.95</td>
<td>5.10</td>
<td></td>
</tr>
<tr>
<td>Crystallisation DPM (×10^5)</td>
<td>13.39</td>
<td>1.43</td>
<td></td>
</tr>
<tr>
<td>DPM mg⁻¹ (×10^4)</td>
<td>1.46</td>
<td>2.81</td>
<td></td>
</tr>
<tr>
<td>Specific activity after dilution</td>
<td>1.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mCi mol⁻¹) before dilution</td>
<td>5.88</td>
<td>5.85</td>
<td></td>
</tr>
</tbody>
</table>

Table 18 Recrystallization to constant activity of the radioactive metabolites, 184, 187 and tripdiolide (1).
2.8 Synthetic studies towards isodehydroabietenolide (91)

The previous section has indicated that the TRP-4a cell system was incapable of converting the allylic alcohol, 171, into tripdiolide (1). Thus, a simpler diterpene precursor, in which the butenolide ring system is not formed, is apparently not utilized in the formation of 1. It seemed appropriate to now consider synthesising precursors in which the butenolide ring system is intact and to subsequently determine whether TRP-4a cell produced enzymes are capable of converting such precursors to the diterpene triepoxides, 1 and 2.

The earlier proposed biosynthetic pathways to tripdiolide (1) envisaged that precursors such as 91 (Schemes 16 and 17) were involved, thereby implying that butenolide ring formation precedes the formation of the triepoxide functionality present in 1 and 2. It was therefore desirable to synthesise the butenolide compound, 91, to test this hypothesis. A synthetic route with the capability of introducing an inexpensive radiolabel into 91 had to be developed. Introduction of a carbon-14 radiolabel in the latter stages of the synthesis was highly desirable.

A previous synthesis of 91 has been carried out by Koike and Tokoroyama which involved the introduction of the C18 lactone carbonyl unit attached to C3 via a [2, 3]-sigmatropic rearrangement of a diterpene analogue appropriately functionalized at C19 (see Scheme 3, 84→85). A similar synthesis of 91 was carried out by van Tamelen et al. The previous syntheses were deemed to be unsuitable for the present study since at no point during the synthesis could a suitable, readily available radiolabel be introduced into the molecule.

The initial approach adopted for this purpose was to introduce a cyanide functionality at C3 of a suitably functionalized derivative of the alkene, 81, for example, 82 (X=OH).
The alkene mixture, 175, was synthesised as previously described from dehydroabietic acid (80) (Scheme 18) and separated by column chromatography using silver nitrate impregnated silica gel to give the alkenes, 188, 189 and 81 in a ratio of 1:2.5:4.5. This was essential since both 189 and 81 would provide access to functionalization at C3 by separate routes. The alkene 81 would also provide a means of functionalization at C19 should an alternative route to the butenolide, 91, be sought.
The alkene, $\text{81}$, was converted to the allylic alcohol, $\text{191}$, via epoxidation using $m$-CPBA and rearrangement of the epoxide, $\text{190}$, using diethylaluminium diisopropylamide.$^{109, 110}$ The same procedure was repeated for the alkene, $\text{189}$, to give the allylic alcohol, $\text{82}$. Conversion of the epoxide, $\text{192}$, proceeded cleanly and in quantitative yield to the allylic alcohol, $\text{82}$, providing a convenient access to functionality at C3. An alternative route to the allylic alcohol, $\text{82}$, was provided by a one-step Sharpless allylic oxidation of the alkene, $\text{81}$, in 68\% yield using selenium dioxide and $t$-butyl hydroperoxide.$^{111}$ The allylic alcohol, $\text{82}$, was synthesised previously by Koike and Tokoroyama following the latter pathway (Scheme 3) $^{45}$ and by Cambie and Franich via the epoxide route.$^{112}$ The two synthetic routes proved to be quite useful since both alkenes $\text{189}$ and $\text{81}$ could be utilized in the synthesis of $\text{82}$. 

\[ \text{81} \xrightarrow{\text{epoxidation}} \text{190} \xrightarrow{\text{rearrangement}} \text{191} \]

\[ \text{189} \xrightarrow{\text{epoxidation}} \text{192} \xrightarrow{\text{rearrangement}} \text{82} \]
The first attempts at introduction of the nitrile involved attempted halogenation at 
C3 by displacement of the hydroxyl group. Reaction of 82 with thionyl chloride resulted in 
the formation of the completely rearranged allylic chloride, 193, in 83% yield. Clearly, the 
displacement of hydroxyl in 82 by halide was to present problems with rearrangement 
and methods were sought in which substitution with halide could be effected without such 
rearrangement taking place.

A review has appeared in the literature which discusses extensively 
the displacement reactions of allylic compounds \(^{113}\) and a number of reactions are 
available which convert allylic alcohols to allylic halides without rearrangement.\(^{114-118}\) 
Reaction of 82 with dimethyl sulphide and N-bromosuccinimide in methylene chloride at 
0°C \(^{115}\) resulted in a 1:1 mixture of the 3-bromo- and 19-bromo- compounds, 194 and 
195 (42%) as an inseparable mixture and the dibromo compound, 196 (26%). A second 
attempt at bromination without rearrangement was carried out using triphenylphosphine 
and carbon tetrabromide in acetonitrile at room temperature.\(^{116}\) The same mixture of 
allylic bromides, 194 and 195 (32%) was obtained along with the elimination product, 
197 (4%) and the starting material (30%). The low yields of allylic bromides and the 
inseparable mixtures rendered the displacement reaction unsuitable for the synthesis of 
the conjugated nitrile.
An alternative method was attempted involving direct displacement of the hydroxyl group with cyanide.\textsuperscript{119} Reaction of 82 with triphenylphosphine and carbon tetrachloride in the presence of sodium cyanide in DMSO resulted in the recovery of starting material even at elevated temperatures (120°C).
Previous work had shown that conversion of the hydroxyl group into a leaving group (tosylate) resulted in predominantly the elimination product, 197.\textsuperscript{120} However an attempt was made to convert the hydroxyl group of 82 into a facile leaving group at low temperature and to trap the ensuing carbocation at C3 with cyanide ion. The reaction was anticipated to proceed via the trifluoromethanesulphonate ester (triflate) of 82 which is a very facile leaving group. Triflate esters of allylic alcohols have been prepared previously in good yield from triflic anhydride and pyridine.\textsuperscript{121, 122} Addition of the allylic alcohol, 82, to a solution of triflic anhydride and pyridine in methylene chloride at -23°C resulted in the complete disappearance of the starting material after 1.5 hours. Subsequent addition of potassium cyanide in DMF resulted not in the expected displacement product, but in formylation of the hydroxyl group to give the formate ester, 198 (24%) and elimination to the diene, 197 (28%).
Since the attempts at introduction of cyanide at C3 of the allylic alcohol, 82, were unsuccessful, due to rearrangements and elimination, introduction of cyanide at a higher level of oxidation of the starting material was investigated. Oxidation of the allylic alcohol, 82, with m-CPBA yielded the epoxy alcohol, 199. Attempted tosylation with toluenesulphonyl chloride in pyridine at 0°C and at room temperature gave no reaction. At elevated temperature (105°C), however, the expected tosylate was not formed, but instead the chlorohydrin, 200 (63%) was the product.

In an attempt to produce and isolate the triflate ester of 199 a reaction was carried out with triflic anhydride and pyridine in methylene chloride at -40°C. TLC analysis of the reaction mixture revealed complete disappearance of the starting material and the appearance of two products. Aqueous workup of the reaction mixture resulted in the isolation of starting material (34%), possibly due to solvolysis of the triflate ester, and the unsaturated rearranged aldehyde, 201 (29%). The same reaction, when repeated in the presence of potassium cyanide and a catalytic amount of crown ether, resulted only in decomposition of the reaction mixture. Attempts at introduction of halide into 199 met with the same fate.
The next avenue of investigation involved the synthesis of the conjugated ketone, 202. An interesting cyanophosphorylation reaction had appeared in the literature involving the formation of a cyanophosphate from a conjugated ketone, followed by Lewis acid catalysed rearrangement and acidic hydrolysis of the phosphate group to give the β-cyano allylic alcohol.†† One of the side products from the reaction sequence was the butenolide ring, formed by simultaneous hydrolysis of the nitrile. Synthesis of the cyanophosphate, 203, was expected to lead to rearrangement, followed by a one step conversion to the butenolide, 91 by acidic hydrolysis of the rearranged cyanophosphate.
Synthesis of the ketone, 202, was achieved from the allylic alcohol, 82, via a Swern oxidation using oxalyl chloride and DMSO at -78°C. Small scale conversion of the allylic alcohol resulted in good yields of the ketone (~80%), but on a larger scale (2.30 g), the yield was much poorer (46%) possibly due to the unstable nature of the conjugated ketone. Conversion of the ketone, 202, with lithium cyanide and diethyl cyanophosphate in DMF gave quite readily the cyanophosphate, 203 (59%). Rearrangement of the cyanophosphate, 203, was attempted under several reaction conditions including refluxing in benzene, addition of boron trifluoride etherate followed by refluxing, and refluxing in toluene. No rearrangement was observed and the starting material was recovered unchanged.

The facile conversion of ketones into nitriles via arenesulphonylhydrazones was seen as a potential route to the introduction of cyanide at C3 of the conjugated ketone, 202. Attempted cyanation using triisopropylsulphonylhydrazide (trisylhydrazide) and potassium cyanide in methanol, followed by thermal decomposition (60°C) of the hydrazone led only to intractable mixtures.
Trisylhydrazide was chosen for the reaction because of its reported facile thermal decomposition\textsuperscript{128, 129} required for its removal after the addition of cyanide. The mild conditions seemed attractive to attempting the reaction on the epoxy ketone, 204. Methanol had to be avoided during the reaction however, because of the potential methanolysis of the epoxide functionality. The epoxy ketone, 204, was synthesised from the epoxy alcohol, 199, by oxidation with PCC. Reaction of the epoxy ketone with trisylhydrazide in THF was carried out at room temperature and monitoring of the reaction by TLC showed complete disappearance of the starting material after 2 hours. Addition of potassium cyanide and a catalytic amount of crown ether, followed by thermal decomposition of the hydrazone at 60°C gave the epoxy cyanohydrin, 205, as an unstable oil which slowly reverted back to the epoxy ketone, 204.

Attention so far had been focused on finding a method of introducing cyanide at C3 since such a route would introduce the desired radiolabel in the form of the relatively cheap and readily available $^{14}$C-labelled cyanide ion. The difficulties
cheap and readily available $^{14}$C-labelled cyanide ion. The difficulties encountered with such a route meant that a different approach to the synthesis of the butenolide, 91, had to be adopted.

A convenient synthesis of butenolides had been developed by Garver and van Tamelen (Scheme 6)\(^4\) involving only 3 steps from the ketone, 107, to the butenolide, 108. The sequence involved the addition of carbon disulphide and conversion to the ketene thioacetal. Addition of methylene via a sulphur ylid and subsequent hydrolysis gave the butenolide, 108. A closer examination revealed that application of this sequence created the potential for the introduction of a radiolabel at either C3 or C4 of the ketone, 206.

\[\text{\includegraphics[width=0.5\textwidth]{206}}\]

A radiolabel could be introduced at C3 via reaction with $^{14}$C carbon disulphide and subsequent conversion to the ketene thioacetal. Alternatively, a $^{14}$C-labelled sulphur ylid is readily available from dimethyl sulphide and $^{14}$C-labelled methyl iodide to give trimethylsulphonium iodide, the immediate precursor to dimethylsulphonium methylide.\(^{131}\)

The synthesis of the butenolide, 91, was carried out according to Scheme 20 in which the alkene, 81, was converted into the ketone, 206, by ozonolysis in methanol-methylene chloride at -78°C followed by treatment with dimethyl sulphide (66% yield). Some benzylic oxidation at C7 was observed yielding the diketone, 207, as a side product (19%). Conversion to the ketene thioacetal, 208, was achieved by addition of the ketone, 206, to a stirred solution of carbon disulphide in THF in the presence of lithium 4-methyl-
2, 6-di-t-butylphenoxide, followed by the addition of methyl iodide at room temperature. The ketene thioacetal, 208, was obtained in near-quantitative yield after chromatography. Treatment of the ketene thioacetal with dimethylsulphonium methylide in THF gave, presumably, an epoxide or mixture of epoxides, which was hydrolysed without isolation to give directly the butenolide, 91, as a light yellow oil in 42% yield after chromatography.

Scheme 20 Synthesis of isodehydroabietenolide (91)

a) O₃, MeOH-CH₂Cl₂ 5:1, -78°C; b) CS₂, 4-Me-2,6-di-t-Bu-C₆H₄OLi, THF, Mel; c) (CH₃)₂S=CH₂, THF, -20°C; d) conc HCl, MeOH-CH₂CN
Since $^{14}$C methyl iodide was relatively cheap in comparison to $^{14}$C carbon disulphide, the route to radiolabel incorporation at C4, via radioactive sulphur ylid, was followed. Incorporation of the radiolabel at C4 was achieved first by synthesis of the $^{14}$C-labelled dimethylsulphonium methylide according to Scheme 21. Dimethyl sulphide was added to 1 mCi of $^{14}$C methyl iodide and the resulting $^{14}$C trimethylsulphonium iodide was allowed to crystallize. Generation of the $^{14}$C ylid was achieved by addition of n-butyllithium to the trimethylsulphonium iodide in THF at -20$^\circ$C. Addition of the ketene thioacetal, 208 and subsequent hydrolysis yielded the butenolide, 209 (6.18 mg, 1.22x$10^7$ DPM), labelled at C19, which was diluted with the inactive butenolide, 91, to 297.68 mg (5.48 mCi mol$^{-1}$). The level of incorporation of the radiolabel was 0.6% which provided a level of radioactivity sufficiently high for the biotransformation studies to proceed using the TRP-4a tissue cultures.

\[
(CH_3)_2S + ^{14}CH_3I \rightarrow (CH_3)_2S^{14}CH_3 I^-
\]

\[
(CH_3)_2S^{14}CH_3 \rightarrow (CH_3)_2S-^{14}CH_2
\]

Scheme 21 Synthesis of radioactive isodehydroabietenolide (209)
2.9 Biotransformation studies of the butenolide, 91, and the radioactive butenolide, 209

Production of the TRP-4a tissue cultures was carried out as previously described by growth in PRD2Co100 growth medium and resuspension in MSNA0.5K0.5 production medium. Preliminary time-course experiments were necessary in order to determine whether the culture cells were able to utilize the butenolide precursor in a biotransformation experiment and to determine the optimum conditions for biotransformation.

2.9.1 Time course studies using the radioactive butenolide, 209

The allylic alcohol, 171, was found to be readily biotransformed over a period of 120 hours at the end of the growth phase. The initial time-course experiments of the butenolide, 209, were based on the previous findings and small scale biotransformations were set up with addition of the precursor at the end of the growth phase; 10 mg samples of 209 were added to the cultures at a concentration of 100 mg L\(^{-1}\). Three experiments involving incubation times of 24, 48 and 72 hours were initiated. At the end of each time period, the cultures were harvested, along with 100 mL of a control sample (no precursor was added) and extracted. In these experiments and in subsequent biotransformation experiments, the spent medium and the cells were not separated and were extracted together. Homogenization of the cultures and extraction into ethyl acetate gave the crude brown extracts according to Table 19.

A blank experiment, involving the addition of the inactive butenolide, 91 (14.89 mg) to 250 mL of the MSNA0.5K0.5 medium without the addition of TRP-4a cells, showed that the butenolide was stable in the medium under the same incubation conditions. The butenolide was recovered unchanged (95%) after 72 hours of incubation with the medium.
Table 19 Reaction conditions for the biotransformation of the radioactive butenolide, 209.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Wt. precursor added (mg)</th>
<th>DPM ($\times 10^5$)</th>
<th>Incubation time (hrs)</th>
<th>Wt. extract (mg)</th>
<th>DPM ($\times 10^5$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.13</td>
<td>4.14</td>
<td>24</td>
<td>52.21</td>
<td>3.41</td>
</tr>
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<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10.05</td>
<td>4.11</td>
<td>48</td>
<td>51.81</td>
<td>3.37</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10.12</td>
<td>4.14</td>
<td>72</td>
<td>51.79</td>
<td>3.44</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TLC analysis of the extracts (toluene-ethyl acetate 4:1), revealed that, in addition to the indigenous metabolites, the radioactive samples contained a metabolite with strong UV activity. The control samples, however, showed no such metabolite under UV. The UV active metabolite was assumed to have arisen from biotransformation of the precursor by the TRP-4a cells. The TLC analysis revealed also the presence of a substantial quantity of the starting material.

Radioactivity profiles of sample 1 (3.20 mg, 2.09$\times 10^4$ DPM) sample 2 (3.77 mg, 2.45$\times 10^4$ DPM) and sample 3 (3.84 mg, 2.54$\times 10^4$ DPM) (see Table 19) were run using toluene-ethyl acetate 4:1 to determine the number of metabolites arising from the precursor and to determine the quantity of precursor used by the cells. The results are presented in Tables 20 to 22 and Figures 29 to 31.
<table>
<thead>
<tr>
<th>Band No.</th>
<th>DPM ($\times 10^3$)</th>
<th>% DPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.64</td>
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</tr>
<tr>
<td>2</td>
<td>2.89</td>
<td>13.8</td>
</tr>
<tr>
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<tr>
<td>5</td>
<td>0.08</td>
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<tr>
<td>6</td>
<td>0.10</td>
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<tr>
<td>7</td>
<td>13.34</td>
<td>63.7</td>
</tr>
<tr>
<td>8</td>
<td>0.19</td>
<td>0.9</td>
</tr>
</tbody>
</table>

**Table 20** Radioactivity profile data for sample 1 (3.20 mg, 2.09x$10^4$ DPM) incubated for 24 hours.

**Figure 29** Radioactivity profile for sample 1 incubated for 24 hours.
<table>
<thead>
<tr>
<th>Band No.</th>
<th>DPM ($\times 10^3$)</th>
<th>% DPM</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.82</td>
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<td>2</td>
<td>2.61</td>
<td>11.8</td>
</tr>
<tr>
<td>3</td>
<td>0.28</td>
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<td>3.82</td>
<td>17.3</td>
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<td>5</td>
<td>0.11</td>
<td>0.5</td>
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<td>6</td>
<td>0.15</td>
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<td>7</td>
<td>14.15</td>
<td>64.1</td>
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<tr>
<td>8</td>
<td>0.15</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Table 21  Radioactivity profile data for sample 2 (3.77 mg, $2.45 \times 10^4$ DPM) incubated for 48 hours.

Figure 30  Radioactivity profile for sample 2 incubated for 48 hours.
<table>
<thead>
<tr>
<th>Band No.</th>
<th>DPM ($\times 10^3$)</th>
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<tbody>
<tr>
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<td>2</td>
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<td>3</td>
<td>0.54</td>
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<tr>
<td>4</td>
<td>4.09</td>
<td>19.6</td>
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<tr>
<td>5</td>
<td>0.09</td>
<td>0.4</td>
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<td>6</td>
<td>0.10</td>
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</tr>
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<td>7</td>
<td>13.67</td>
<td>65.4</td>
</tr>
<tr>
<td>8</td>
<td>0.09</td>
<td>0.4</td>
</tr>
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</table>

Table 22  Radioactivity profile data for sample 3 (3.84 mg, $2.54 \times 10^4$ DPM) incubated for 72 hours.

Figure 31  Radioactivity profile for sample 3 incubated for 72 hours
The radioactivity profiles show that a substantial quantity of the starting material (65%) remained unchanged even after 72 hours of incubation, indicating that either the concentration of the precursor was too high and/or that the incubation time was too short. In addition to the UV active component, the radioactivity profiles indicate that at least one other metabolite was present in the extracts, arising from the radioactive precursor. The level of the metabolite is seen to decrease on increasing the incubation time from 24 hours to 48 and 72 hours, while the level of the UV active metabolite is seen to increase. This observation suggests that the metabolite is being further biotransformed by the cells to the UV active metabolite.

Another small scale experiment was conducted in which the radioactive precursor, 209, was added to the TRP-4a culture 11 days after resuspension in MSNA0.5K0.5 production medium. Previous studies had shown that production of triptolide by the TRP-4a cultures began to increase dramatically after day 11 following resuspension of the cells from the PRD2Co100 growth medium to the MSNA0.5K0.5 production medium. The precursor, 209 (10.61 mg, 4.34×10^5 DPM), was added to the culture (200 mL) at day 11 after resuspension in MSNA0.5K0.5 and incubated until the end of the growth phase (15 days after the addition of the precursor). Harvesting and extraction were carried out as previously described to give a crude brown extract (176.90 mg, 3.43×10^5 DPM). Radioactivity profiles were carried out using toluene-ethyl acetate 4:1 (9.52 mg of sample, 1.85×10^5 DPM) and methylene chloride-ethyl acetate 1:1 (8.04 mg of sample, 1.56×10^4 DPM) as the eluants.

The radioactivity profile run in toluene-ethyl acetate 4:1 (Table 23, Figure 32) shows that the precursor was almost completely utilized by the cells with approximately 4% remaining unmetabolized. The UV active metabolite was the major metabolite (43%) while at least two other metabolites were indicated (18% and 20%). The radioactivity profile run with methylene chloride-ethyl acetate 1:1 (Table 24, Figure 33) shows that very little radioactivity was associated with the triptolide fraction. Triptolide (2) was present in band 5 but was masked by the presence of other metabolites.
<table>
<thead>
<tr>
<th>Band No.</th>
<th>DPM ($\times 10^3$)</th>
<th>% DPM</th>
</tr>
</thead>
<tbody>
<tr>
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<td>3.17</td>
<td>18.3</td>
</tr>
<tr>
<td>2</td>
<td>3.38</td>
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<td>3</td>
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</tr>
<tr>
<td>4</td>
<td>7.48</td>
<td>43.3</td>
</tr>
<tr>
<td>5</td>
<td>0.27</td>
<td>1.6</td>
</tr>
<tr>
<td>6</td>
<td>0.10</td>
<td>0.6</td>
</tr>
<tr>
<td>7</td>
<td>0.66</td>
<td>3.8</td>
</tr>
<tr>
<td>8</td>
<td>0.05</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Table 23  Radioactivity profile data for the culture extract incubated for 15 days with the butenolide, and eluted with toluene-ethyl acetate 4:1

Figure 32  Radioactivity profile of the culture extract incubated with the butenolide for 15 days, and eluted with toluene-ethyl acetate 4:1
<table>
<thead>
<tr>
<th>Band No.</th>
<th>DPM ($\times 10^3$)</th>
<th>% DPM</th>
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<tbody>
<tr>
<td>1</td>
<td>1.32</td>
<td>10.0</td>
</tr>
<tr>
<td>2</td>
<td>0.35</td>
<td>2.7</td>
</tr>
<tr>
<td>3</td>
<td>1.42</td>
<td>10.7</td>
</tr>
<tr>
<td>4</td>
<td>1.08</td>
<td>8.1</td>
</tr>
<tr>
<td>5</td>
<td>2.05</td>
<td>15.5</td>
</tr>
<tr>
<td>6</td>
<td>7.00</td>
<td>53.0</td>
</tr>
</tbody>
</table>

Table 24 Radioactivity profile data for the culture extract incubated for 15 days with the butenolid, and eluted with methylene chloride-ethyl acetate 1:1.

Figure 33 Radioactivity profile of the culture extract incubated with the butenolid for 15 days, and eluted with methylene chloride-ethyl acetate 1:1.
Large scale biotransformation of the radioactive butenolide, 209, and the inactive butenolide, 91

The latter conditions (addition at approximately day 11 after resuspension in MSNA_{0.5}K_{0.5}) were chosen to carry out the large scale biotransformation in which the radioactive butenolide, 209 and the inactive butenolide, 91 were incubated concurrently with the TRP-4a cultures. The radioactive butenolide, 209 (101.26 mg, 4.14×10^6 DPM) was incubated with 2 L of the culture while the inactive butenolide, 91, was incubated with 6 L of the culture. Addition took place 12 days after resuspension of the cells in MSNA_{0.5}K_{0.5} medium. The cultures were then incubated until the end of the growth phase (20 days after the addition of the precursor) and harvested and extracted.

Extraction of the radioactive culture yielded 781.83 mg (2.93×10^6 DPM, 94.4%) of an orangey-brown extract (one flask (500 mL) of the radioactive culture was found to be contaminated and was therefore discarded; the % DPM recovered is calculated from the remaining DPM of 3.11×10^6 DPM). The inactive culture yielded 2.56 g of an orangey-brown extract.

Isolation of the radioactive metabolites from the extract was achieved by column chromatography and monitoring of the radioactivity of the crude fractions. Partial separation of the radioactive extract by column chromatography using toluene-ethyl acetate 4:1 and rapid elution with ethyl acetate and methanol gave 8 crude fractions. Separation of fraction 1 by column chromatography (hexanes-ethyl acetate 4:1) gave the starting material, 209 (13.01 mg, 4.53×10^5 DPM), while separation of fractions 2 and 3 yielded the benzylic ketone, 210 (36.42 mg, 1.31×10^6 DPM) as a light yellow foam with a strong UV activity. Fractions 4, 5 and 6 were separated by column chromatography using methylene chloride-ethyl acetate 5:1 to give 5 fractions, the first 3 of which were separated further by preparative TLC using toluene-ethyl acetate-chloroform-formic acid 35:15:16:1 to give the hydroxy ketone, 211 (5.19 mg, 1.37×10^5 DPM), and the benzylic alcohol, 212 (8.07 mg, 2.21×10^5 DPM). The two remaining fractions, from the latter
column chromatography, were separated by preparative TLC using benzene-methanol-acetic acid 85:10:5 to give the C2 hydroxy compound, 213 (3.69 mg, 1.58×10^5 DPM) and tripdiolide (1, 2.72 mg, 2.97×10^4 DPM). The results are summarized in Table 25 and the separation procedure is summarized in Figure 34.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Wt. (mg)</th>
<th>DPM (×10^5)</th>
<th>% DPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>209</td>
<td>13.01</td>
<td>4.53</td>
<td>19.6</td>
</tr>
<tr>
<td>210</td>
<td>36.42</td>
<td>13.10</td>
<td>56.7</td>
</tr>
<tr>
<td>211</td>
<td>5.19</td>
<td>1.37</td>
<td>5.9</td>
</tr>
<tr>
<td>212</td>
<td>8.07</td>
<td>2.21</td>
<td>9.6</td>
</tr>
<tr>
<td>213</td>
<td>3.69</td>
<td>1.58</td>
<td>6.8</td>
</tr>
<tr>
<td>1</td>
<td>2.72</td>
<td>0.30</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Table 25  Weights and activities of the isolated radioactive metabolites.
Crude extract (781.83 mg, $2.93 \times 10^6$ DPM)

Column chromatography toluene-EtOAc 4:1, EtOAc

1 2 3 4 5 6 7 8

Column chromatography hexanes-EtOAc 4:1

209 13.01 mg, $4.53 \times 10^2$ DPM

Column chromatography toluene-EtOAc 9:1

210 36.42 mg, $1.31 \times 10^6$ DPM

TLC toluene-EtOAc-CHCl$_3$-HCO$_2$H 35:15:16:1

211 5.19 mg, $1.37 \times 10^5$ DPM

TLC benzene-MeOH-AcOH 85:10:5

212 8.07 mg, $2.21 \times 10^5$ DPM

213 4.69 mg, $1.58 \times 10^5$ DPM

1 2.72 mg, $2.97 \times 10^5$ DPM

Figure 34 Chromatographic separation of 209, 210, 211, 212, 213 and tripdiolide (1) from the radioactive extract
Isolation of the corresponding inactive metabolites for identification was achieved by column chromatography using toluene-ethyl acetate 4:1 and rapid elution with ethyl acetate to give seven crude fractions. Column chromatography of fraction 1 (hexanes-ethyl acetate 4:1) yielded the starting material, 91 (64.13 mg), while separation of fraction 2 by preparative TLC (methylene chloride-ethyl acetate 5:1) yielded the benzylic ketone, 214 (104.36 mg). Fractions 5 and 6 were separated by column chromatography using methylene chloride-ethyl acetate 5:1 followed by preparative TLC using toluene-ethyl acetate-chloroform-formic acid 35:15:16:1 to give the hydroxy ketone, 215 (9.61 mg) and the benzylic alcohol, 216 (9.35 mg), and separation using benzene-methanol-acetic acid 85:10:5 yielded the C2 hydroxy compound, 96 (13.35 mg). The structures are given in Scheme 22 below. The results are summarized in Table 26 and the chromatographic separation is summarized in Figure 35.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Wt. (mg)</th>
<th>% of recovered material</th>
</tr>
</thead>
<tbody>
<tr>
<td>91</td>
<td>64.13</td>
<td>31.9</td>
</tr>
<tr>
<td>214</td>
<td>104.36</td>
<td>52.0</td>
</tr>
<tr>
<td>215</td>
<td>9.61</td>
<td>4.8</td>
</tr>
<tr>
<td>216</td>
<td>9.35</td>
<td>4.7</td>
</tr>
<tr>
<td>96</td>
<td>13.35</td>
<td>6.6</td>
</tr>
</tbody>
</table>

Table 26 Isolated yields of the inactive metabolites.
Figure 35 Chromatographic separation of 91, 214, 215, 216 and 96 from the inactive extract

The structure of 216 was confirmed by synthesis from the ketone, 214. Reduction of the ketone with sodium borohydride yielded a compound with identical spectra, mp and
TLC characteristics with the alcohol, 216. A $^1\text{H}^{-1}\text{H}$ COSY spectrum of 96 confirmed the regiochemistry of the hydroxyl group at C2 (Figure 36). Two cross peaks at $\delta$ 5.17 correspond to the coupling of H2α to the protons at C1 (2/1, 2/1') which show only a cross peak at $\delta$ 2.57 corresponding to geminal coupling (1/1'). An nOe difference spectrum confirmed the stereochemistry of the hydroxyl group. Irradiation of H2α at $\delta$ 5.17 showed no enhancement of the C10 methyl group at $\delta$ 1.26 and likewise, irradiation of the C10 methyl group showed no enhancement of the H2α signal at $\delta$ 5.17.

The radioactivity profiles from the initial time-course studies (Figures 29 to 31) suggest that the sequence of biotransformation proceeds via hydroxylation at C7 to give 216, followed by oxidation to the ketone, 214. Further biotransformation to the hydroxy ketone, 215, is suggested in Scheme 22 to proceed either via hydroxylation of 214 or via oxidation of the C2 hydroxyl metabolite, 96.
Figure 36 COSY spectrum of 96
The specific activities of the metabolites, 210, 212 and 213 were determined by recrystallization to constant activity. The ketone, 210, was first converted to its 2,4-dinitrophenylhydrazone derivative, 217 (36.71 mg, 8.27×10^5 DPM), and recrystallized from ethyl acetate.

The hydroxy compound, 212, was recrystallized from ether-hexanes. Dilution with inactive material, 216 (5.41 mg) was necessary after the first recrystallization. The C2 hydroxy compound, 213, was recrystallized from hexanes-ethyl acetate after dilution with 6.15 mg of inactive material, 96. Recrystallization of tripdiolide (1) from ethanol after dilution with an authentic sample, yielded zero activity after the first recrystallization (Table 27).

Due to the instability of the hydroxy ketone, 211, it was not possible to carry out recrystallizations to determine the specific activity. The fraction of the extract containing triptolide (2) contained no significant activity and was investigated no further.

The results presented in Table 27 indicate that no incorporation of radioactive butenolide, 209, into tripdiolide (1) had taken place during the biotransformation experiment. The isolation of the metabolites, 210 to 213 (and the corresponding inactive metabolites, 214 to 96) show, however, that the butenolide precursor experienced enzymatic oxidations at positions C2 and C7, similar to those observed in tripdiolide.
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>217</th>
<th>212</th>
<th>213</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wt. (mg)</td>
<td>36.71</td>
<td>8.07</td>
<td>10.84</td>
<td>8.34</td>
</tr>
<tr>
<td>DPM (×10^5)</td>
<td>8.27</td>
<td>2.21</td>
<td>1.58</td>
<td>0.30</td>
</tr>
<tr>
<td>DPM mg⁻¹ (×10^4)</td>
<td>2.25</td>
<td>2.73</td>
<td>1.46</td>
<td>0.36</td>
</tr>
<tr>
<td>1st</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Wt. (mg)</td>
<td>22.81</td>
<td>7.59</td>
<td>8.04</td>
<td>4.21</td>
</tr>
<tr>
<td>Crystallisation</td>
<td>5.52</td>
<td>0.68</td>
<td>1.15</td>
<td>0.00</td>
</tr>
<tr>
<td>DPM (×10^5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPM mg⁻¹ (×10^4)</td>
<td>2.42</td>
<td>0.90</td>
<td>1.43</td>
<td>0.00</td>
</tr>
<tr>
<td>2nd</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wt. (mg)</td>
<td>13.21</td>
<td>3.42</td>
<td>4.46</td>
<td></td>
</tr>
<tr>
<td>Crystallisation</td>
<td>3.29</td>
<td>0.37</td>
<td>0.65</td>
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<tr>
<td>DPM (×10^5)</td>
<td></td>
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<td></td>
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<tr>
<td>DPM mg⁻¹ (×10^4)</td>
<td>2.49</td>
<td>1.07</td>
<td>1.46</td>
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<tr>
<td>3rd</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wt. (mg)</td>
<td>9.23</td>
<td>1.13</td>
<td>1.81</td>
<td></td>
</tr>
<tr>
<td>Crystallisation</td>
<td>2.24</td>
<td>0.12</td>
<td>0.26</td>
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<td>DPM (×10^5)</td>
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<tr>
<td>DPM mg⁻¹ (×10^4)</td>
<td>2.43</td>
<td>1.07</td>
<td>1.45</td>
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<td>4th</td>
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<tr>
<td>Wt. (mg)</td>
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<td>DPM (×10^5)</td>
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</tr>
<tr>
<td>DPM mg⁻¹ (×10^4)</td>
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<tr>
<td>Specific activity</td>
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<td>(mCi mol⁻¹)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before dilution</td>
<td>5.49</td>
<td>5.24</td>
<td>4.72</td>
<td></td>
</tr>
</tbody>
</table>

Table 27 Recrystallization to constant activity of the radioactive metabolites, 217, 212, 213 and tripdiolide (1).

1 The first recrystallisation yielded 2.18 mg (6.81×10⁴ DPM) which was diluted with unlabelled material, 216 (5.41 mg) in order to continue the recrystallisation.
2.10 Conclusions

The lack of incorporation of the butenolide, 209, into the diterpene triepoxides, 1 and 2, and the observed oxidations suggest that, while the precursor may be reaching the site of biosynthesis of the diterpene triepoxides, activation of ring C of the butenolide...
might be necessary before biotransformation into tripdiolide can take place. That is, biosynthesis of abietane-type precursors must occur with hydroxylation at ring C (most likely at C14). It appears also, that the lactone ring must be present in ring A before oxidation at C2 and C7 can occur (cf. the allylic alcohol, 171, in which oxidations at these positions did not occur). The above observations are made bearing in mind that, although no incorporation into the diterpene triepoxides was obtained, changes in experimental parameters, such as time of addition of the precursor, length of incubation, culture medium used etc., might also yield the desired biotransformations. That is, the conditions used in the present study might not be the optimum conditions necessary for the presence of all the enzyme systems required for biotransformation of the precursor. However, a possible biosynthetic pathway based on these results is presented in Scheme 23, in which hydroxylation of ring C occurs prior to formation of the butenolide, 35. Subsequent enzymatic oxidation at C7 and epoxidation are postulated to yield the triepoxide system of the diterpene triepoxides.
3 EXPERIMENTAL

TRP-4a tissue culture production was carried out by Gary Hewitt, Gin Lee, Roger Suen, David Chen and Fay Hutton of the Biological Services, Chemistry Department, University of British Columbia. The cultures were grown in glass or in steel air-lift fermentors (Microferm, New Brunswick Scientific or an in-house fabricated fermentor, respectively). The spent medium was freeze-dried using a Dura-Dry freeze-dryer with a Dura-Top Bulk Tray Dryer (FTS Systems Inc.).

$^1$H NMR spectra were run at 300 MHz and 400 MHz using Varian XL 300 and Bruker WH 400 spectrometers, respectively. Tetramethylsilane was used as the internal standard and all peaks are recorded in ppm ($\delta$) relative to TMS ($\delta$ 0.00 ppm). $^{13}$C NMR spectra were run at 75.3 MHz using a Varian XL 300 spectrometer using the $^{13}$C signals of the deuterated solvents as the internal standards.

Low resolution mass spectra were recorded using Kratos MS 50 and MS 80 mass spectrometers. High resolution mass spectra were run on a Kratos MS 50 mass spectrometer. Chemical ionization mass spectra were recorded on a Delsi-Nermag R10-10C mass spectrometer using isobutane as the carrier gas.

IR spectra were recorded on a Perkin Elmer 710B infrared spectrophotometer, and Fourier transform IR spectra were recorded on a Perkin Elmer 1710 infrared Fourier transform spectrophotometer. Optical rotations were recorded on a Perkin Elmer 141 polarimeter. UV spectra were recorded on a Unicam SP 800B ultraviolet spectrophotometer.

All melting points were recorded on a Reichert melting point apparatus or a Fisher-Johns melting point apparatus and are uncorrected.

Column chromatography was carried out using Merck silica gel 60, 230-400 Å mesh; while analytical and preparative TLC was performed using Merck pre-coated silica gel 60 F$_{254}$ TLC plates. Radioactivity profiles were carried out using Merck pre-coated silica gel F$_{254}$ TLC plates (0.25 mm) with concentrating zone. Vacuum liquid
chromatography (VLC) was carried out using Merck silica gel 60G in sintered glass funnels with medium glass frits. Preparative HPLC separations were performed on a Waters Associates Prep LC/System 500 installed with PrepPAK-500/silica cartridges and a refractive index detector. All solvents used for chromatography were BDH Omnisolv glass distilled spectro-grade solvents.

Radioactivity was measured using a Beckman LS 9000 Liquid Scintillation Counter with a Beckman LS 9000 data reduction program and was recorded in disintegrations per minute (DPM). All samples were prepared in Wheaton disposable glass scintillation vials using 10 mL of Fisher Scintiverse E Universal LSC Cocktail as the scintillant. $^{14}$C Paraformaldehyde was purchased from Amersham Canada Ltd. and $^{14}$C methyl iodide was purchased from Sigma Chemical Company.

Synthetic samples were visualized on analytical TLC plates (Merck) by UV and by spraying with a 5% solution of ammonium molybdate in 10% sulphuric acid, followed by heating at 125°C until blue spots developed. Tissue culture extracts were visualized on TLC by spraying first with a 30% solution of concentrated sulphuric acid in glacial acetic acid, then with a 5% solution of anisaldehyde in isopropanol, followed by heating at 125°C for approximately 10 minutes.

Elemental analyses were carried out by Mr. P. Borda of the Microanalytical Laboratory, University of British Columbia, Vancouver.

X-ray analyses were carried out by Dr. Steve Rettig, Chemistry Department, University of British Columbia and were determined using a Nonius CAD 4 and a Rigaku AFC 6 X-ray spectrometers.
3.1 Growth conditions for the TRP-4a tissue cultures

The cultures were grown and maintained (as outlined in *Planta Medica* 51) in PRD$_2$Co$_{100}$ medium at 26°C for 22 days and then resuspended as a 10% inoculum in MSNA$_{0.5}$K$_{0.5}$ production medium. The cultures were incubated at 26°C in air-lift fermentors for 33 to 48 days before harvesting. The cells were harvested by filtration through miracloth and were frozen until the time of extraction. The spent medium was freeze-dried and stored frozen until the time of extraction.

3.2 Extraction of the TRP-4a tissue cultures

3.2.1 General extraction procedure

**Cells:** The cells were thawed and homogenized in a Waring blender with ethyl acetate (1 L). The cells were then filtered using celite, and washed with ethyl acetate (300 mL). The filtrate was separated and the aqueous layer was extracted with ethyl acetate (3×200 mL) and discarded. The ethyl acetate layers were combined, dried over sodium sulphate (anhydrous) and the solvent was evaporated. The orange-brown extract was dried under vacuum and kept frozen until further use.

The filtered cell homogenate was suspended in methanol (500 mL) and sonicated for 2 hours. Filtration and solvent evaporation gave a brown gummy extract which was dried under vacuum and kept frozen until further use.
**Spent medium**: The freeze-dried spent medium was reconstituted in water (300-500 mL) and extracted with ethyl acetate (3×200 mL). The organic layers were combined, dried over sodium sulphate (anhydrous) and the solvent was evaporated. The dark brown extract was dried under vacuum and frozen until further use. The aqueous layer was evaporated and the residue was sonicated with methanol (200 mL) and filtered. The filtrate was evaporated and the brown extract was dried under vacuum and frozen until further use. Extraction of batches TRP#221, 222, 224 and 225 and the weights of the extracts are given in Tables 1 and 2.

3.3 Preparation of samples for bioassay

Samples of the crude TRP#221 culture extracts were forwarded for immunological assay and for antifertility assay. The solids obtained from the ethyl acetate extracts were prepared in a manner so as to generate emulsions in water (*vide infra*) while the solids obtained from the methanol extracts were forwarded without further preparation.

The ethyl acetate cell extract (0.84 g) from batch TRP#221 was dissolved in ethanol (15 mL) and polyvinylpyrrolidone (PVP, 4.20 g) was added. The solvent was evaporated and the residue was dried under vacuum to give 5.03 g of a water emulsifiable solid. The procedure was repeated with the ethyl acetate spent medium extract (0.44 g, with 2.19 g of PVP) to give 2.65 g of a water emulsifiable solid.

3.4 Partial separation of the ethyl acetate extracts for biological assay

3.4.1 **Cell extracts**

The remaining ethyl acetate cell extracts (from batches TRP#222, 224 and 225), were combined (3.73 g) and separated into 8 partially purified fractions by vacuum liquid
chromatography using Merck silica gel 60G (80 g) packed into a sintered glass funnel (150 mL, medium frit), attached to a bell jar with a side-arm, which was connected to a water aspirator. Fifty mL fractions were collected and stepwise elution was carried out using: i) hexanes-ethyl acetate 4:1 (6×50 mL); ii) hexanes-ethyl acetate 3:1 (6×50 mL); iii) hexanes-ethyl acetate 2:1 (6×50 mL); iv) hexanes-ethyl acetate 1:1 (6×50 mL); and v) ethyl acetate (6×50 mL). The column was flushed with ethyl acetate-methanol 1:1 (250 mL) to elute the polar material.

Elution with hexanes-ethyl acetate 4:1 yielded an oily fraction (378.76 mg) containing fatty acid-like compounds (fraction 1). Further elution with hexanes-ethyl acetate 4:1 and hexanes-ethyl acetate 3:1 yielded fraction 2 (389.66 mg), containing non-polar diterpenes and sterols. Elution with hexanes-ethyl acetate 3:1 and 2:1 yielded fraction 3 (273.60 mg) as a brown solid containing several terpenoid components. Further elution with hexanes-ethyl acetate 2:1 gave fraction 4 (267.59 mg) while elution with hexanes-ethyl acetate 1:1 yielded fraction 5 (505.63 mg). Both fractions contained triterpenoid components. Elution with ethyl acetate gave fraction 6 (291.01 mg) as a light brown solid containing polar triterpenes. Elution with ethyl acetate and ethyl acetate-methanol 1:1 gave fraction 7 (175.80 mg) as a light brown solid containing polar triterpenes. Fraction 8 (1.22 g) was eluted with ethyl acetate-methanol 1:1 yielding polar material as a dark brown solid.

3.4.2 Spent medium extracts:

The remaining ethyl acetate spent medium extracts (from batches TRP#222, 224 and 225) were combined (1.17 g) and partially separated by VLC using a sintered glass funnel (60 mL, medium frit, Merck silica gel 60G, 30 g). Thirty mL fractions were collected to give 10 partially purified fractions. Stepwise elution was carried out using: i) hexanes (5×30 mL); ii) hexanes-ethyl acetate 19:1 (5×30 mL); iii) hexanes-ethyl acetate 9:1
(5×30 mL); iv) hexanes-ethyl acetate 4:1 (5×30 mL); v) hexanes-ethyl acetate 1:1 (5×30 mL) and vi) ethyl acetate (5×30 mL). The polar material was eluted from the column using ethyl acetate-methanol 1:1 (150 mL).

Elution with hexanes and hexanes-ethyl acetate 19:1 gave fraction 1 (4.32 mg) as a yellow solid containing non-polar terpenoid components. Elution with hexanes-ethyl acetate 19:1, 9:1 and 4:1 gave fraction 2 (23.89 mg) and elution with hexanes-ethyl acetate 4:1 gave fraction 3 (14.28 mg). Both fractions contained several terpenoid components. Further elution with hexanes-ethyl acetate 4:1 and 1:1 yielded fraction 4 (60.64 mg) as a light brown solid containing diterpenoid and triterpenoid metabolites. Elution with hexanes-ethyl acetate 1:1 and ethyl acetate yielded fraction 5 (128.52 mg) as a brown solid containing diterpenoids and triterpenoids. Elution with ethyl acetate gave fraction 6 (206.21 mg) as a yellow-brown solid containing triptolide (2) and several triterpenes. Further elution with ethyl acetate yielded fraction 7 (204.75 mg) as a yellow-brown solid containing tripdiolide (1) and several triterpenes. Elution with ethyl acetate and ethyl acetate-methanol 1:1 gave fraction 8 (52.43 mg) as a brown solid containing triterpenes and polar material. Elution with ethyl acetate-methanol 1:1 gave fraction 9 (303.69 mg) while further elution with ethyl acetate-methanol 1:1 yielded fraction 10 (76.54 mg) as polar material in the form of brown solids.

3.5 Preparation of the partially purified extracts for biological assay

All the fractions were treated as above with PVP (5:1 PVP: fraction, w/w) in order to obtain samples capable of forming emulsions in water. The samples were then forwarded for immunological and antifertility assays.
3.6 Isolation of pure metabolites from the TRP-4a tissue cultures

3.6.1 Growth and extraction of further batches of TRP-4a cultures

The growth of the cells was carried out as in Section 3.1. To obtain large quantities of cells and spent medium, 6 batches (10 to 17 L) were grown in bioreactors for 25 to 39 days to afford a total of 78 L (Table 3). The batches were combined on harvesting and filtered through miracloth. The cells (wet weight 8.94 kg) were frozen and the spent medium (60.6 L) was concentrated to 1.4 L by freeze-drying.

3.6.2 Extraction of the cells

At the time of extraction, the cells were thawed, homogenized in a Waring blender with ethyl acetate (4.5 L) and filtered through celite. The cell debris was washed with ethyl acetate (1.5 L) and discarded. The filtrate was separated and the aqueous layer (4.0 L) was extracted with ethyl acetate (1.0 L) and discarded. The organic layers were combined dried over sodium sulphate (anhydrous) and the solvent was evaporated to give 13.08 g of a dark orange extract.

3.6.3 Extraction of the spent medium

The concentrated spent medium (1.4 L) was diluted to 1.7 L and extracted with ethyl acetate (3×1.0 L). The organic layers were filtered through celite to destroy the emulsions and the celite was washed with ethyl acetate (0.5 L). The organic layers were combined, dried over sodium sulphate (anhydrous) and the solvent was evaporated to give 4.73 g of an orange- brown extract.

3.6.4 Chromatographic separation of the cell extract metabolites

Initial purification of the cell extract (13.08 g) was carried out by filtration through silica gel (Merck silica gel 230-400 Å, 60 mL) using ethyl acetate, to remove the polar material. Evaporation of the solvent yielded 9.04 g of crude extract as an orange solid.
Isolation of the triterpene acids (137-139, 51):

**Method A:** The cell extract (3.01 g) was partially separated by VLC using a 150 mL sintered glass funnel (medium frit, Merck silica gel 60G, 90 g). Stepwise elution using: i) benzene (5×30 mL); ii) benzene-acetone 4:1 (25×30 mL); iii) benzene-acetone 3:1 (15×30 mL); iv) benzene-acetone 2:1 (18×30 mL) and v) ethyl acetate (300 mL) gave 10 fractions. Fractions 1 (349.11 mg) and 2 (45.70 mg) were colourless oily fractions consisting of fatty acid-like compounds. Fraction 3 (1.17 g), a dark orange fraction, contained diterpenes, sterols, triterpenes and quinone methides (see below for the separation of this fraction). Fraction 4 yielded oleanolic acid (127, 432.26 mg). Fraction 5 (162.39 mg) consisted of triterpenes 137 and 138 and fraction 6 (147.96 mg) contained triterpenes 137, 138, 139 and 51. Fraction 7 (92.80 mg) contained triterpenes 138, 139 and 51, while fraction 8 (75.03 mg) contained a small quantity of triterpene 139 and triterpene 51. Fractions 9 and 10 (347.58 mg and 52.42 mg respectively) consisted of triterpene 51 and some polar material.

Separation of fractions 5, 6, 7 and 8 was carried out by preparative TLC (Merck silica 60G, 6×0.5 mm silica gel plates) using methylene chloride-methanol-acetic acid 100:2:1 (the plates were eluted 4 times) to give triterpene 137 (27.25 mg), triterpene 138 (65.77 mg), triterpene 139 (20.85 mg) and triterpene 51 (21.80 mg). Fractions 9 and 10 were combined and washed with methanol to give a further quantity of 51 (157.13 mg).

In summary, chromatographic separation yielded oleanolic acid (127, 432.26 mg) and triterpenes 137 (27.25 mg), 138 (65.77 mg), 139 (20.85 mg) and 51 (157.13 mg).

**Method B:** 5.83 g of the cell extract was partially separated by VLC using a 150 mL sintered glass funnel (medium frit, Merck silica gel 60G, 90 g). Stepwise elution was carried out using: i) benzene (5×30 mL); ii) benzene-acetone 4:1 (15×30 mL); iii) benzene-acetone 3:1 (10×30 mL); iv) benzene-acetone 2:1 (5×30 mL); v) benzene-acetone 1:1 (10×30 mL) and ethyl acetate (250 mL), yielding 10 crude fractions. Fraction 1 (756.14 mg) was an oily fraction consisting of fatty acid-like compounds. Fraction 2
(2.38 g) containing diterpenes, sterols, triterpenes and quinone methides was subsequently combined with fraction 3 from Method A for further separation (*vide infra*). Fraction 3 (126.58 mg) was a mixture of oleanolic acid (127) and triterpene 137, while fraction 4 (281.26 mg) contained triterpenes 137 and 138. Fractions 5 and 6 (74.93 mg and 62.50 mg respectively) consisted of mainly triterpene 138 and a small quantity of triterpene 139. Fraction 7 (219.67 mg) contained triterpenes 138 and 139, while fraction 8 (106.61 mg) contained triterpenes 138, 139, and 51. Fractions 9 and 10 (569.53 mg and 534.98 mg respectively) contained triterpene 51 and polar material.

Fractions 3 to 8 were combined and separated by column chromatography (Merck silica gel 230-400 Å, methylene chloride-methanol-acetic acid 100:2:1) to give oleanolic acid (127, 101.97 mg), a mixture of 137 and 138 (101.97 mg), triterpene 138 (167.69 mg), triterpene 139 (181.17 mg) and triterpene 51 (85.34 mg). The mixture of 137 and 138 was separated by further column chromatography (Merck silica gel 230-400Å, methylene chloride-methanol-acetic acid 100:1:1) to give 137 (55.80 mg) and a further quantity of 138 (121.74 mg). Fractions 9 and 10 were combined and washed with methanol to give 51 (359.65 mg).

In summary, chromatographic separation via Method B yielded oleanolic acid (127, 101.97 mg) and triterpenes 137 (55.80 mg), 138 (289.43 mg), 139 (181.17 mg) and 51 (444.99 mg).

22β-Hydroxy-3-oxoolean-12-en-29-oic acid (137):

Colourless prisms (ethyl acetate); mp 268-270°C; [α]_D^22 +92.9° (c 0.70, MeOH); UV (MeOH) \( \lambda_{max} \) 217 (ε 726); IR (CHCl_3) cm\(^{-1}\) 3618 (OH), 2938 (CH), 2640(OH, br), 1698 (C=O), 1463, 1386, 1217, 724; \(^1\)H NMR (CDCl_3) δ 0.91 (3H, s, CH_3), 1.04 (3H, s, CH_3), 1.07 (3H, s, CH_3), 1.09 (3H, s, CH_3), 1.11 (3H, s, CH_3). 1.15 (3H, s, CH_3), 1.41 (3H, s, CH_3), 0.86 - 2.32 (m, aliphatic H), 2.39 (1H, ddd, H2α, J = 4, 6, 16 Hz), 2.55 (1H, ddd, H2β, J = 4, 11, 16 Hz), 3.50 (1H, s, OH), 3.58 (1H, dd, H22α, J = 3.5, 7.0 Hz), 5.33 (1H, t, H12); \(^13\)C NMR (CD_3OD) δ 15.4, 17.2, 19.9, 21.1, 21.8, 24.0, 25.3, 25.4, 26.4, 26.7,
29.1, 32.7, 34.5, 36.9, 38.0, 38.2, 39.4, 39.9, 41.7, 42.6, 44.9, 47.1, 47.5, 55.3, 75.4, 122.9, 144.2, 181.6, 217.8; MS m/z (rel. intensity) 470 (3.8), 452 (7.0), 437 (2.7), 426 (4.4), 424 (5.5), 408 (15.7), 391 (3.2), 340 (11.1), 325 (4.1), 264 (43.7), 246 (67.8), 217 (79.9), 205 (57.3), 201 (38.3), 189 (46.0), 171 (44.5), 159 (42.7), 147 (55.9), 135 (82.3), 119 (95.1), 107 (90.5), 81 (89.8), 69 (71.4), 55 (100), 43 (53.4); HRMS calcd. for C$_{30}$H$_{46}$O$_{4}$: 470.3398; found: 470.3414; Anal. calcd. for C$_{30}$H$_{46}$O$_{4}$: C 76.55, H 9.85; found C 76.02, H 9.97.

22α-Hydroxy-3-oxoolean-12-en-29-oic acid (138):

Colourless prisms (MeOH); mp 289-290°C; [$\alpha$]$^D_{22}$ +84.4° (c 1.09, MeOH); UV (MeOH) $\lambda_{\text{max}}$ 212 (ε 1938); IR (CHCl$_3$) cm$^{-1}$ 3615 (OH), 2976 (CH), 2630 (OH, br), 1698 (C=O), 1461, 1386, 1219, 1027, 910, 715; $^1$H NMR (CDCl$_3$) δ 1.02 (3H, s, CH$_3$), 1.04 (3H, s, CH$_3$), 1.07 (3H, s, CH$_3$), 1.08 (3H, s, CH$_3$), 1.10 (3H, s, CH$_3$), 1.18 (3H, s, CH$_3$), 1.29 (3H, s, CH$_3$), 1.26 - 2.27 (m, aliphatic H), 2.38 (1H, ddd, H2α, J = 4, 6, 16 Hz), 2.55 (1H, ddd, H2β, J = 4, 11, 16 Hz), 3.60 (1H, dd, H22β J = 4, 12 Hz), 5.29 (1H, t, H12); $^{13}$C NMR (CDCl$_3$) δ 15.3, 16.7, 19.2, 19.7, 20.4, 21.6, 23.7, 24.5, 25.5, 26.1, 26.5, 32.0, 34.2, 36.5, 36.6, 38.3, 39.3, 39.6, 39.9, 42.2, 42.7, 46.3, 46.8, 47.5, 55.2, 75.3, 123.1, 142.6, 183.3, 217.8; MS m/z (rel. intensity) 470 (0.6), 452 (11.8), 437 (2.0), 408 (1.5), 391 (1.3), 353 (0.6), 340 (0.5), 326 (1.7), 299 (1.2), 285 (2.2), 264 (3.7), 246 (100.0), 228 (12.2), 218 (25.1), 205 (35.8), 201 (20.0), 185 (20.9), 173 (14.2), 159 (16.9), 145 (23.6), 131 (31.0), 119 (38.0), 107 (29.3), 95 (34.3), 81 (22.1), 69 (14.4), 55 (24.6), 43 (21.7); HRMS calcd. for C$_{30}$H$_{46}$O$_{4}$: 470.3398; found: 470.3417; Anal. calcd. for C$_{30}$H$_{46}$O$_{4}$: C 76.55, H 9.85; found C 76.40, H 9.90.

3β, 22β-Dihydroxyolean-12-en-29-oic acid (139):

Colourless prisms (MeOH-H$_2$O); mp 281-283°C; [$\alpha$]$^D_{22}$ +55.0° (c 0.60, MeOH); UV (MeOH) $\lambda_{\text{max}}$ 216 (ε 835); IR (KBr) cm$^{-1}$ 3467 (OH), 3422 (OH, br), 2976 (CH), 2640 (OH, br), 1698 (C=O), 1471, 1393, 1298, 1240, 1048, 1032, 1005; $^1$H NMR (CD$_3$N)
δ 0.76 (3H, s, CH₃), 0.85 (6H, s, 2CH₃), 1.02 (3H, s, CH₃), 1.03 (3H, s, CH₃), 1.08 (3H, s, CH₃), 1.63 (3H, s, CH₃), 0.62 - 2.63 (m, aliphatic H), 3.22 (1H, dd, H3α, J = 6, 9.5 Hz), 3.80 (1H, brd, H22α, J = 4.5 Hz), 5.21 (1H, t, H12); ¹³C NMR (CD₅N) δ 15.8, 16.6, 17.2, 18.7, 21.0, 23.8, 24.9, 25.5, 26.3, 28.0, 28.7, 28.8, 30.7, 33.2, 37.2, 37.7, 38.0, 39.1, 39.3, 39.9, 41.5, 42.4, 44.6, 48.0, 55.7, 75.3, 77.9, 123.1, 144.2, 181.0; MS m/z (rel. intensity) 472 (6.1), 454 (5.0), 439 (2.6), 426 (2.0), 411 (2.1), 393 (1.5), 264 (99.7), 246 (53.0), 231 (15.4), 217 (100.0), 207 (59.9), 190 (45.2), 175 (39.3), 161 (22.7), 147 (37.9), 135 (74.1), 119 (49.9), 107 (48.2), 95 (52.4), 81 (49.9), 69 (48.6), 55 (48.7), 43 (30.7); HRMS calcd. for C₃₀H₄₈O₄: 472.3554; found: 472.3567; Anal. calcd. for C₃₀H₄₈O₄·H₂O: C 73.43, H 10.27; found: C 73.53, H 10.40.

3β, 22α-Dihydroxyolean-12-en-29-oic acid (51):

Colourless prisms (MeOH); mp 293-298°C (subl.); [α]D +93.5° (c 0.46, MeOH); UV (MeOH) λmax 212 (ε 1509); IR (KBr) cm⁻¹ 3475 (OH), 3387 (OH, br), 2946 (CH), 2550 (OH, br), 1698 (C=O), 1467, 1384, 1235, 1038; ¹H NMR (CD₅N) δ 0.98 (3H, s, CH₃), 1.03 (3H, s, CH₃), 1.04 (3H, s, CH₃), 1.23 (3H, s, CH₃), 1.28 (3H, s, CH₃), 1.37 (3H, s, CH₃), 1.58 (3H, s, CH₃), 0.82 - 2.74 (m, aliphatic H), 3.42 (1H, dd, H3α, J = 6, 10 Hz), 4.01 (1H, dd, H22β, J = 5.5, 13 Hz), 5.38 (1H, t, H12); ¹³C NMR (CD₅N) δ 15.8, 16.6, 17.0, 18.8, 19.9, 21.4, 23.9, 25.4, 26.1, 26.3, 28.0, 28.7, 32.9, 37.2, 38.6, 39.1, 39.4, 40.2, 41.2, 42.4, 47.2, 47.9, 55.6, 74.7, 78.0, 123.1, 144.2, 181.1; MS m/z (rel intensity) 472 (0.2), 454 (2.6), 436 (1.7), 421 (1.3), 410 (0.6), 393 (1.2), 342 (1.1), 325 (0.6), 314 (0.9), 299 (2.0), 264 (2.7), 246 (100.0), 218 (21.6), 207 (18.1), 201 (15.5), 190 (35.6), 175 (20.3), 159 (14.8), 145 (20.1), 131 (22.5), 119 (32.2), 107 (23.2), 95 (25.9), 81 (17.7), 69 (12.2), 55 (14.7); HRMS calcd for C₃₀H₄₈O₄: 472.3554; found: 472.3540; Anal. calcd. for C₃₀H₄₈O₄¹: C 76.23, H 10.23; found: C 76.10, H 10.33.

¹All the data for this compound were obtained on a sample which was recrystallized from ethanol, except for the X-ray analysis. Recrystallization from methanol was shown by X-ray analysis to yield crystals as the 1:1 methanol solvate.
**Oleanolic acid (127):**

Colourless prisms (MeOH); mp 268-269.5°C; [α]$_D^{22}$ +58.1° (c 0.74, CHCl$_3$); IR (CHCl$_3$) cm$^{-1}$ 3600 (OH), 2925 (CH), 1686 (C=O), 1462, 1380, 1227, 1200, 930, 710; $^1$H NMR (CDCl$_3$) δ 0.79 (3H, s, CH$_3$), 0.86 (3H, s, CH$_3$), 0.94 (3H, s, CH$_3$), 0.97 (3H, s, CH$_3$), 1.00 (3H, s, CH$_3$), 1.15 (3H, s, CH$_3$), 1.24 (3H, s, CH$_3$), 0.73 - 2.22 (m, aliphatic H), 3.24 (1H, dd, H$_3$α, J = 4, 10 Hz), 5.24 (1H, t, H12); $^{13}$C NMR (C$_5$D$_5$N) δ 16.1, 17.0, 17.4, 19.1, 20.4, 24.2, 26.5, 26.8, 27.5, 28.4, 28.8, 29.1, 30.2, 33.1, 33.3, 36.8, 37.6, 39.4, 39.7, 40.4, 41.8, 42.2, 43.1, 46.8, 48.2, 55.9, 78.2, 123.2, 144.7, 181.4; MS m/z (rel. intensity) 456 (1.6), 438 (1.0), 423 (1.6), 411 (1.2), 395 (0.6), 377 (0.6), 342 (0.6), 301 (1.3), 248 (100.0), 233 (11.0), 207 (18.4), 203 (14.9), 187 (77.4), 173 (80.8), 161 (18.2), 147 (22.5), 133 (18.6), 119 (26.5), 107 (23.4), 95 (32.9), 81 (26.3), 69 (18.7), 55 (22.2); HRMS calcd. for C$_{30}$H$_{48}$O$_3$: 456.3605; found: 456.3606.

**Reduction of 22α-hydroxy-3-oxoolean-12-en-29-oic acid (138):**

The acid, 138 (11.24 mg, 0.02 mmol) was dissolved in ethanol (1 mL) and sodium borohydride (4.52 mg, 0.12 mmol) was added. The reaction mixture was stirred at room temperature for 30 minutes and ethyl acetate (30 mL) was added. The solution was washed with 10% HCl (2×5 mL) and water (2×10 mL) and the solvent was evaporated to give 8.63 mg (76.4%) of 51 as a white solid (TLC, mp, $^1$H NMR and MS were identical with isolated 51).

**Reduction of 22β-hydroxy-3-oxoolean-12-en-29-oic acid (137):**

The acid, 137 (6.18 mg, 0.01 mmol) was treated as above with sodium borohydride, which yielded, after work-up, 5.92 mg (95.3%) of 139 as a white solid (TLC, mp, $^1$H NMR and MS were identical with isolated 139).
Esterification of 137, 138, 139 and 51:

Suspensions of the triterpenes 137, 138, 139 and 51 in ether (3mL) were treated with an ethereal solution of excess diazomethane at room temperature overnight. Evaporation of the solvent gave the methyl esters 140, 141, 142 and 143 as white solids:

Methyl-22β-hydroxy-3-oxoolean-12-en-29-oate (140):

White solid; mp 195-197°C; [α]_D^{22} +82.3° (c 1.15, CHCl₃); IR (CHCl₃) cm⁻¹ 3425 (OH, br), 2910 (CH), 1719 (C=O), 1698 (C=O), 1232, 1205, 938, 800, 675; 1H NMR (CDCl₃) δ 0.90 (3H, s, CH₃), 1.04 (3H, s, CH₃), 1.06 (3H, s, CH₃), 1.08 (3H, s, CH₃), 1.10 (3H, s, CH₃), 1.14 (3H, s, CH₃), 1.36 (3H, s, CH₃), 0.82 - 2.27 (m, aliphatic H), 2.38 (1H, ddd, H2α, J = 4, 6, 16 Hz), 2.55 (1H, ddd, H2β, J = 4, 11, 16 Hz), 3.55 (1H, dd, H22α, J = 3, 4 Hz), 3.69 (3H, s, OCH₃), 5.33 (1H, t, H12); MS m/z (rel. intensity) 484 (42.5), 469 (4.6), 466 (7.9), 453 (10.4), 425 (16.9), 407 (4.7), 391 (3.5), 375 (6.3), 278 (100.0), 265 (57.1), 260 (25.7), 247 (43.3), 231 (83.2), 219 (35.5), 205 (40.5), 200 (41.7), 187 (27.9), 171 (58.1), 161 (16.9), 144 (40.7), 125 (60.7), 112 (37.7), 95 (26.7), 81 (20.1), 69 (16.7), 55 (15.7); HRMS calcd. for C_{31}H_{48}O_{4}: 484.3552; found: 484.3554.

Methyl-22α-hydroxy-3-oxoolean-12-en-29-oate (141):

White solid; mp 149-151°C; [α]_D^{22} +92.9° (c 0.85, CHCl₃); IR (CHCl₃) cm⁻¹ 3600 (OH), 2930 (CH), 1718 (C=O), 1698 (C=O), 1459, 1382, 1218, 1119, 790, 715, 670; 1H NMR (CDCl₃) δ 1.02 (3H, s, CH₃), 1.04 (3H, s, CH₃), 1.07 (3H, s, CH₃), 1.08 (3H, s, CH₃), 1.11 (3H, s, CH₃), 1.18 (3H, s, CH₃), 1.26 (3H, s, CH₃), 1.35 - 2.23 (m, aliphatic H), 2.38 (1H, ddd, H2α, J = 4, 6, 16 Hz), 2.55 (1H, ddd, H2β, J = 4, 11, 16 Hz), 3.59 (1H, dd, H22β, J = 4, 12 Hz), 3.68 (3H, s, OCH₃), 5.28 (1H, t, H12), MS m/z (rel intensity) 484 (18.5), 466 (5.0), 452 (21.2), 437 (3.4), 406 (3.4), 391 (3.5), 340 (2.3), 326 (4.1), 299 (1.9), 278 (43.3), 265 (6.8), 260 (13.3), 246 (100.0), 231 (28.9), 218 (18.2), 205 (29.8),
185 (21.6), 171 (23.0), 159 (13.1), 147 (21.0), 131 (19.2), 120 (22.3), 107 (17.8), 95 (27.9), 81 (10.5), 69 (1.9), 55 (9.1); HRMS calcd. for C$_{31}$H$_{48}$O$_4$: 484.3552; found: 484.3570.

Methyl-3β, 22β-dihydroxyolean-12-en-29-oate (142):

White solid; mp 228-229°C; [α]$^2_0$ +64.0° (c 0.88, CHCl$_3$); IR (CHCl$_3$) cm$^{-1}$ 3665 (OH), 3586 (OH), 2900 (CH), 1718 (C=O), 1481, 1430, 1381, 1218, 1119, 735; 1H NMR (CDCl$_3$) $\delta$ 0.80 (3H, s, CH$_3$), 0.90 (3H, s, CH$_3$), 0.96 (3H, s, CH$_3$), 0.99 (3H, s, CH$_3$), 1.01 (3H, s, CH$_3$), 1.13 (3H, s, CH$_3$), 1.36 (3H, s, CH$_3$), 0.73 - 2.27 (m, aliphatic H), 3.23 (1H, dd, H3α, J = 4, 10 Hz), 3.55 (1H, dd, H22α, J = 4, 8 Hz), 3.68 (3H, s OCH$_3$), 5.30 (1H, t, H12); MS m/z (rel. intensity) 486 (13.3), 471 (1.2), 468 (3.0), 455 (3.6), 427 (2.0), 409 (1.5), 354 (1.4), 330 (1.4), 304 (1.3), 292 (3.4), 278 (100.0), 260 (23.6), 246 (23.3), 231 (79.5), 219 (23.3), 207 (47.9), 200 (47.3), 189 (25.6), 171 (60.2), 144 (43.0), 135 (34.8), 119 (37.3), 112 (37.6), 107 (28.1), 95 (27.3), 81 (23.1), 69 (17.0), 55 (18.5); HRMS calcd. for C$_{31}$H$_{50}$O$_4$: 486.3711; found: 486.3722.

Methyl-3α, 22α-dihydroxyolean-12-en-29-oate (143):

White solid; mp 118-121°C; [α]$^2_0$ +74.9° (c 0.90, CHCl$_3$); IR (CHCl$_3$) cm$^{-1}$ 3655 (OH), 3600 (OH), 2932 (CH), 1718 (C=O), 1465, 1385, 1235, 1205, 1120, 1033, 798, 715; 1H NMR (CDCl$_3$) $\delta$ 0.80 (3H, s, CH$_3$), 0.94 (3H, s, CH$_3$), 0.98 (3H, s, CH$_3$), 1.00 (6H, s, 2CH$_3$), 1.17 (3H, s, CH$_3$), 1.26 (3H, s, CH$_3$), 0.73 - 2.22 (m, aliphatic H), 3.23 (1H, dd, H3α, J = 4, 10 Hz), 3.58 (1H, dd, H22β, J = 4, 12 Hz), 3.67 (3H, s, OCH$_3$), 5.26 (1H, t, H12); MS m/z (rel. intensity) 486 (3.1), 468 (1.1), 454 (4.7), 436 (1.9), 421 (1.2), 409 (0.7), 393 (1.0), 342 (0.8), 328 (1.1), 314 (0.9), 300 (1.7), 278 (16.9), 246 (100.0), 231 (14.9), 218 (17.6), 207 (24.1), 190 (35.0), 175 (15.6), 159 (11.9), 147 (15.8), 131 (16.1),
119 (20.6), 107 (14.5), 95 (22.5), 81 (9.3), 69 (7.5), 55 (7.7); HRMS calcd. for C_{31}H_{50}O_{4}: 486.3711; found: 486.3695.

**Methyl-3, 22-dioxoolean-12-en-29-oate (144):**

Excess pyridinium chlorochromate was added to a stirred solution of 142 (10.00 mg, 0.02 mmol) in methylene chloride (1.0 mL) and the reaction mixture was stirred at room temperature for 30 minutes. The reaction mixture was filtered through florisil (30 mL) and the florisil was washed with ether. The washing and the filtrate were combined and the solvent was evaporated to give 8.23 mg (83.0%) of 144 as a white solid. mp 180.5-182°C; [α]_{D}^{22} +5.7° (c 4.75 CHCl₃); IR (CHCl₃) cm⁻¹ 3000 (CH), 2940 (CH), 1721 (C=O), 1698 (C=O), 1462, 1386, 1218, 1120, 935, 760; ¹H NMR (CDCl₃) δ 1.03 (3H, s, CH₃), 1.04 (3H, s, CH₃), 1.07 (3H, s, CH₃), 1.09 (3H, s, CH₃), 1.11 (3H, s, CH₃), 1.16 (3H, s, CH₃), 1.24 (3H, s, CH₃), 1.21 - 2.11 (m, aliphatic H), 2.32 (1H, dd, H19β, J = 3.5, 15 Hz), 2.39 (1H, ddd, H2α, J = 4, 6, 16 Hz), 2.48 (1H, d, H21β, J = 14.5 Hz), 2.56 (1H, ddd, H2β, J = 4, 11, 16 Hz), 2.99 (1H, d, H21α, J = 14.5 Hz), 3.71 (3H, s, OCH₃), 5.38 (1H, t, H12), MS m/z (rel. intensity) 482 (18.4), 467 (2.3), 464 (2.7), 451 (1.8), 423 (2.0), 405 (1.6), 339 (1.7), 298 (0.8), 276 (100.0), 263 (11.1), 247 (16.4), 215 (17.6), 205 (13.5), 199 (16.7), 187 (13.8), 173 (9.8), 159 (9.9), 142 (25.5), 134 (24.3), 119 (20.5), 114 (68.7), 105 (13.2), 93 (9.4), 81 (8.8), 69 (4.9), 55 (10.3); HRMS calcd. for C_{31}H_{46}O_{4}: 482.3398; found: 482.3405.

**Oxidation of 143:**

Excess pyridinium chlorochromate was added to a stirred solution of 143 (5.03 mg, 0.01 mmol) in methylene chloride (1.0 mL) and the reaction mixture was stirred at room temperature for 30 minutes. The reaction mixture was filtered through florisil (30 mL) and
the florisil was washed with ether. The washings and the filtrate were combined and the solvent was evaporated to give 3.76 mg (75.4%) of 144 as a white solid.

Separation and identification of the metabolites from fraction 3 (from Method A) and fraction 2 (from Method B)

Fraction 3 (1.17 g) from Method A and fraction 2 (2.38 g) from Method B (vide supra) were combined (3.55 g) and partially separated by column chromatography (Merck silica gel 230-400 Å, 350 g). Stepwise elution was carried out using: i) hexanes (250 mL); ii) hexanes-ethyl acetate 9:1 (500 mL); iii) hexanes-ethyl acetate 4:1 (1500 mL); iv) hexanes-ethyl acetate 3:1 (1000 mL); v) hexanes-ethyl acetate 2:1 (1000 mL) and vi) ethyl acetate (1000 mL) to give 12 crude fractions. Fractions A and B (126.30 mg and 178.25 mg respectively) were complex mixtures of minor components and were not further investigated, while fractions C and D (198.84 mg and 49.34 mg respectively) consisted of two major components (see below for the separation). Fractions E and F (489.19 mg and 64.06 mg respectively) yielded β-sitosterol (128), while fractions G, H and I (69.81 mg, 46.35 mg and 38.15 mg respectively) were complex mixtures and were not further investigated. Fractions J and K (815.16 mg and 195.71 mg respectively) contained orange-coloured quinone methides and polpunonic acid (55) (see below for the separation) and fraction L yielded oleanolic acid (127, 564.65 mg).

In summary, chromatographic separation yielded several crude fractions, β-sitosterol (128, 553.25 mg) and oleanolic acid (127, 564.65 mg).

β-Sitosterol (128):

Colourless needles (ethyl acetate-hexanes); mp 132-134°C; IR (CHCl₃) cm⁻¹ 3600 (OH), 2950 (CH), 1600, 1465, 1437, 1380, 1202, 1052, 1021, 790, 702; ¹H NMR (CDCl₃) δ 0.69 (3H, s, CH₃), 0.82 (3H, d, CH₃), 0.86 (3H, t, CH₃), 0.93 (3H, d, CH₃),
0.99 (3H, d, CH₃), 1.02 (3H, s, CH₃), 0.93 - 2.33 (m, aliphatic H), 3.53 (1H, m, H₃α), 5.36 (1H, brd, H6); MS m/z (rel. intensity) 414 (100.0), 399 (38.6), 396 (58.8), 381 (42.8), 367 (10.2), 354 (8.6), 341 (5.0), 329 (51.4), 314 (91.5), 303 (51.8), 299 (23.1), 281 (29.4), 273 (44.6), 255 (46.6), 231 (45.8), 229 (45.3), 213 (66.1), 199 (23.3), 187 (20.8), 173 (29.5), 161 (52.4), 145 (65.6), 133 (46.3), 121 (44.8), 119 (44.1), 107 (76.1), 95 (71.6), 81 (70.3), 69 (57.3), 55 (86.8), 43 (70.6); HRMS calcd. for C₂₉H₅₀O: 414.3864; found: 414.3866. The above spectral data were found to be identical with those of an authentic sample of β-sitosterol.

Separation of fractions C and D:

Fractions C and D were subjected to preparative TLC (Merck silica gel 60 F₂₅₄, 2mm) using methylene chloride-ethyl acetate 19:1 (the plate was eluted twice) and a further separation using methylene chloride-hexanes 1:1 (the plate was eluted 3 times), to give an inseparable mixture (121.81 mg) of 2 compounds which spectral data suggests a mixture of α-amyrin (145) and β-amyrin (146), and the diterpene 147 (49.68 mg).

α-Amyrin (145) and β-amyrin (146):

White solid; IR (CHCl₃) cm⁻¹ 3445 (OH), 2930 (CH), 1638 (C=C), 1461, 1385, 1218, 1037; ¹H NMR (CDCl₃) δ 0.75 (s, CH₃), 0.81 (d, 2CH₃), 0.84 (s, CH₃), 0.87 (s, CH₃), 0.88 (s, 2CH₃), 0.90 (s, CH₃), 0.94 (s, CH₃), 0.97 (s, 2CH₃), 0.99 (s, CH₃), 1.00 (s, CH₃), 1.13 (s, CH₃), 0.70 - 2.17 (m, aliphatic H), 3.23 (m, H₃α), 3.48 (m, H₃α), 5.11 (m, H12), 5.19 (m, H12); ¹³C NMR (CDCl₃) δ 13.0, 13.8, 14.6, 15.5, 15.6, 16.8, 17.7, 18.3, 18.3, 21.2, 21.8, 23.5, 23.7, 24.1, 24.9, 25.5, 25.8, 26.0, 26.1, 26.3, 26.9, 27.2, 27.3, 27.7, 28.1, 28.2, 28.4, 29.7, 29.8, 30.2, 31.1, 32.1, 32.3, 32.6, 32.7, 33.3, 33.8, 33.9, 34.7, 34.8, 35.7, 35.8, 36.1, 37.0, 37.1, 38.5, 38.6, 46.8, 47.2, 47.6, 48.6, 50.3, 52.0, 52.7, 55.1, 65.7, 78.7, 79.0, 79.2, 105.9, 110.8, 117.8, 121.7, 125.3, 130.8, 145.3, 145.9; MS m/z (rel
intensity) 426 (8.6), 411 (16.5), 393 (7.6), 286 (3.1), 271 (5.2), 257 (10.2), 218 (100.0), 203 (52.1), 189 (29.9), 175 (20.1), 161 (15.8), 147 (19.0), 135 (31.8), 121 (32.3), 109 (47.7), 95 (59.3), 81 (49.9), 69 (76.1), 55 (61.8); HRMS calcd. for C$_{30}$H$_{50}$O: 426.3864; found: 426.3849.

12-Methoxyabieta-8, 11, 13-trien-3α-ol (147):

Colourless needles (hexanes); mp 157-158°C; [α]$_D^{22}$ +44° (c 0.72, CHCl$_3$); IR (CHCl$_3$) cm$^{-1}$ 3620 (OH), 2951 (CH), 1488, 1417, 1335, 1230, 1165, 1132, 1095, 775, 718, 678; $^1$H NMR (CDCl$_3$) δ 0.96 (3H, s, C4 CH$_3$), 1.05 (3H, s, C4 CH$_3$), 1.20 (3H, s, C10 CH$_3$), 1.20 (3H, d, CH(CH$_3$)$_2$, J = 7 Hz), 1.21 (3H, d, CH(CH$_3$)$_2$, J = 7 Hz), 1.26 - 2.17 (m, aliphatic H), 2.76 (1H, ddd, H7α, J = 2, 7, 16 Hz), 3.02 (1H, dd, H7β, J = 7, 16 Hz), 3.28 (1H, septet, H15), 3.51 (1H, brt, H3β, W$^J$ = 7 Hz), 3.72 (3H, s, OCH$_3$), 7.04 (1H, s, H11), 7.05 (1H, s, H14); $^{13}$C NMR (CDCl$_3$) δ 18.2, 22.1, 23.9, 24.0, 24.7, 24.8, 25.9, 26.0, 28.1, 29.7, 31.7, 37.7, 43.3, 60.2, 75.5, 120.4, 123.7, 128.5, 137.9, 148.9, 154.8; MS m/z (rel intensity) 316 (20.9), 301 (4.7), 283 (100.0), 241 (4.6), 215 (5.1), 199 (3.6), 189 (6.9), 173 (5.7), 159 (4.1), 147 (4.8), 129 (4.5), 115 (3.9), 91 (4.1), 81 (2.0), 69 (3.4), 55 (5.6), 43 (9.8); HRMS calcd. for C$_{21}$H$_{32}$O$_2$: 316.2404; found: 316.2411.

Separation of fractions J and K:

Fractions J (815.16 mg) and K (195.71 mg) were combined (1.01 g) and partially separated by column chromatography (Merck silica gel 230-400 Å, 100 g). Gradient elution was carried out using: i) methylene chloride-ethyl acetate 19:1 (750 mL); ii) methylene chloride-ethyl acetate 9:1 (700 mL) and iii) ethyl acetate (200 mL) to give 9 fractions. Fractions 1 and 2 (13.18 mg and 36.17 mg respectively) contained a major orange-coloured compound and a minor compound, while fraction 3 (62.11 mg) consisted of two orange-coloured compounds. Fractions 4 and 5 (25.97 mg and 21.21 mg
respectively) consisted of a major orange-coloured compound and a minor component. Fractions 6 and 7 (28.69 mg and 232.43 mg respectively) showed 2 compounds on TLC, while fractions 8 and 9 (411.67 mg and 40.97 mg respectively) consisted of one major compound and several minor compounds.

Separation of fractions 1 and 2 by preparative TLC (Merck silica F$_{254}$, 0.5 mm, chloroform-methanol 98:2, followed by a further separation using toluene-ethyl acetate-chloroform-formic acid 35:15:16:1 yielded tingenone (148, 15.30 mg) and the friedelane methyl ester, 149 (13.62 mg). Separation of fraction 3 by preparative TLC (Merck silica F$_{254}$, 0.5 mm, toluene-ethyl acetate-chloroform-formic acid 35:15:16:1, plate eluted twice) gave tingenone (148, 14.00 mg) and 22β-hydroxytingenone (150, 13.04 mg). Fractions 4 and 5 were separated by preparative TLC (Merck silica F$_{254}$, 0.5 mm, chloroform-methanol 98:2) to yield 22β-hydroxytingenone (150, 15.55 mg) and an unidentified mixture (9.12 mg). Column chromatography of fraction 7 (Merck silica gel 230-400 Å, 25 g, methylene chloride-ethyl acetate 5:1) gave the dihydroxy triterpene, 151 (50.75 mg), an unidentified mixture (27.27 mg) and the triterpene, 152 (28.48 mg). Column chromatography of fractions 8 and 9 (Merck silica gel 230-400 Å, 50 g, methylene chloride-ethyl acetate 5:1) gave polpunonic acid (55, 247.13 mg).

In summary, chromatographic separation yielded tingenone (148, 29.30 mg), the friedelane methyl ester, 149 (13.62 mg), 22β-hydroxytingenone (150, 28.59 mg), The dihydroxy triterpenes, 151 (50.75 mg) and 152 (28.48 mg) and polpunonic acid (55, 247.13 mg).

**Tingenone (148):**

Orange crystals (ethyl acetate-hexanes); mp 140-142°C (dec); [α]$_D^{22}$ -82.6° (c 0.84, CHCl$_3$); UV (CHCl$_3$) $\lambda_{max}$ 225 (ε 5128) 256 (ε 8204) 424 (ε 8718); IR (CHCl$_3$) cm$^{-1}$ 3530 - 3536 (OH), 2935 (CH), 1701 (C=O), 1645 (C=O, w), 1600 (C=C), 1548, 1512, 1441, 1380, 1292, 1208, 1090, 916, 790, 748, 672; $^1$H NMR (CDCl$_3$) δ 0.99 (3H, s, CH$_3$),
1.00 (3H, d, C20 CH₃), 1.02 (3H, s, CH₃), 1.35 (3H, s, CH₃), 1.52 (3H, s, CH₃), 2.23 (3H, s, C4 CH₃), 0.79 - 2.29 (m, aliphatic H), 1.87 (1H, d, H22β, J = 14 Hz), 2.50 (1H, m, H20α), 2.92 (1H, d, H22α, J = 14 Hz). 6.39 (1H, d, H7, J = 8 Hz), 6.56 (1H, d, H1, J = 1 Hz), 6.99 (1H, br, OH), 7.05 (1H, dd, H6, J = 1, 8 Hz); ¹³C NMR (CDCl₃) δ 10.3, 15.1, 19.7, 21.5, 28.5, 29.9, 32.0, 32.5, 33.7, 35.4, 38.2, 39.1, 40.6, 41.9, 42.7, 43.4, 44.6, 52.5, 117.2, 118.0, 119.8, 127.6, 133.9, 146.0, 164.7, 168.7, 178.2, 213.8; MS m/z (rel intensity) 420 (86.8), 406 (25.5), 405 (22.4), 391 (5.6), 378 (6.6), 342 (14.1), 330 (10.7), 295 (7.6), 283 (7.3), 267 (11.9), 253 (39.3), 241 (100.0), 227 (43.9), 213 (24.6), 202 (72.9), 201 (70.2), 187 (25.8), 175 (16.4), 163 (21.3), 135 (23.7), 121 (33.1), 109 (39.9), 95 (48.0), 81 (36.9), 69 (41.6), 55 (61.5); HRMS calcd. for C₂₈H₃₆O₃: 420.2666; found: 420.2681.


Yellow crystals (EtOH); mp 250-251°C; [α]₂²²D +101.4° (c 0.22, MeOH); UV (MeOH) λ max 203 (ε 1810); IR (CHCl₃) cm⁻¹ 3460 (OH, br), 3010, 2950 (CH), 1725 (C=O, sh), 1710 (C=O), 1700 (C=O, sh), 1205, 995, 927, 785, 720, 670; ¹H NMR (CDCl₃) δ 0.80 (3H, s, CH₃), 0.84 (3H, s, CH₃), 0.91 (3H, d, CH₃), 0.94 (3H, s, CH₃), 1.07 (3H, d, CH₃), 1.36 (3H, s, CH₃), 3.64 (3H, s, OCH₃), 1.16 - 1.85 (m, aliphatic H), 2.02 (1H, m), 2.15 - 2.38 (5H, m), 2.60 - 2.65 (2H, m) 2.77 (1H, m, H20α), 3.66 (1H, s, OH), 4.61 (1H, brd, H22α); MS m/z (rel. intensity) 486 (100.0), 471 (3.1), 468 (5.2), 426 (75.2), 411 (7.6), 399 (16.7), 398 (16.7), 385 (19.5), 357 (5.7), 340 (13.4), 317 (27.0), 257 (27.5), 231 (15.0), 217 (29.5), 203 (14.0), 189 (16.7), 177 (17.5), 163 (21.2), 149 (25.8), 135 (36.5), 121 (81.1), 109 (67.7), 95 (55.2), 81 (51.3), 67 (44.2), 55 (60.6); HRMS calcd. for C₃₀H₄₆O₅: 486.3343; found: 486.3352.

22β-Hydroxytingenone (I50):

Orange crystals (benzene-hexanes); mp 220-224°C (dec); [α]₂²²D -484.6° (c 0.13, CHCl₃); UV (CHCl₃) λ max 233 (ε 4678), 254 (ε 6683), 264 (ε 5346), 426 (ε 10469); IR
(CHCl₃) cm⁻¹ 3450 (OH), 2950 (CH), 1701 (C=O), 1648 (C=O, w), 1598 (C=O), 1548, 1512, 1441, 1380, 1292, 1210, 1112, 1090, 1000, 908, 740, 670; ¹H NMR (CDCl₃) δ 0.88 (3H, s, CH₃), 0.99 (3H, s, CH₃), 1.08 (3H, d, C20 CH₃, J = 7 Hz), 1.37 (3H, s, CH₃), 1.52 (3H, s, CH₃), 2.23 (3H, s, C4 CH₃), 1.26 - 2.30 (m, aliphatic H), 2.67 (1H, m, H20α), 3.67 (1H, br, OH), 4.56 (1H, s, H22α), 6.41 (1H, d, H7, J = 8 Hz), 6.56 (1H, d, H1, J = 1 Hz), 7.00 (1H, d, H6, J = 1, 8 Hz); ¹³C NMR (CDCl₃) δ 10.3, 14.7, 20.5, 21.5, 25.0, 28.2, 29.5, 29.9, 32.0, 33.9, 39.1, 40.6, 40.8, 42.7, 44.3, 44.8, 44.9, 76.3, 117.3, 118.0, 119.9, 127.6, 134.0, 146.0, 164.7, 168.7, 178.2, 213.3; MS m/z (rel. intensity) 436 (43.4), 422 (9.5), 403 (2.2), 342 (4.2), 330 (5.2), 253 (12.8), 241 (20.8), 227 (14.0), 217 (8.7), 202 (100.0), 187 (12.1), 135 (7.9), 121 (10.1), 107 (10.8), 95 (13.1), 81 (11.6), 69 (13.6), 55 (18.3); HRMS calcd. for C₂₆H₃₆O₄: 436.2615; found: 436.2620.

3β, 29-Dihydroxyolean-12-ene (151):

Colourless prisms (EtOH); mp 241-242°C; [α]⁺²² +115.9° (c 0.82, MeOH); IR (KBr) cm⁻¹ 3294 (OH), 2946 (CH), 1466, 1381, 1095, 1072, 1041, 997, 656; ¹H NMR (CDCl₃) δ 0.80 (3H, s, CH₃), 0.85 (3H, s, CH₃), 0.91 (3H, s, CH₃), 0.94 (3H, s, CH₃), 0.97 (3H, s, CH₃), 1.00 (3H, s, CH₃), 1.14 (3H, s, CH₃), 0.73 - 2.02 (m, aliphatic H), 3.22 (1H, dd, H3α J = 3.5, 11 Hz), 3.27 (2H, d, H29, J = 5.5 Hz), 5.21 (1H, t, H12); MS m/z (rel. intensity) 442 (6.1), 427 (1.8), 411 (9.6), 393 (0.5), 288 (0.7), 273 (1.0), 234 (100.0), 219 (8.7), 203 (21.1), 201 (21.3), 187 (21.6), 175 (13.1), 161 (8.1), 147 (14.3), 135 (18.5), 119 (19.1), 107 (19.8), 95 (28.0), 81 (22.9), 69 (18.0), 55 (21.9); HRMS calcd. for C₃₀H₅₀O₂: 442.3813; found: 442.3805.

3β, 11α-Dihydroxyolean-12-ene (152):

Colourless crystals (EtOH); mp 230-234°C; [α]⁺²² +55.3° (c 1.14, CHCl₃); IR (CHCl₃) cm⁻¹ 3675 (OH), 3605 (OH), 2938 (CH), 1601, 1471, 1390, 1370, 1214, 1039, 1001, 980, 940, 718, 675; ¹H NMR (CDCl₃) δ 0.83 (3H, s, CH₃), 0.86 (3H, s, CH₃), 0.91 (6H, s, 2CH₃), 1.03 (6H, s, 2CH₃), 1.08 (3H, s, CH₃), 1.23 (3H, s, CH₃), 0.80 - 2.10 (m,
aliphatic H), 3.26 (1H, dd, H3α, J = 5, 10 Hz) 4.21 (1H, dd, H11β, J = 2.5, 7.5 Hz), 5.27 (1H, d, H12, J = 2.5 Hz); 13C NMR (CDCl3) δ 15.5, 16.9, 18.1, 18.5, 19.8, 23.6, 26.2, 26.7, 27.3, 28.1, 28.5, 31.1, 32.3, 33.1, 33.3, 34.6, 37.0, 38.0, 39.0, 40.4, 41.8, 43.4, 46.5, 46.5, 55.2, 56.5, 67.6, 78.7, 125.4, 149.5; MS m/z (rel. intensity) 442 (4.3), 424 (94.1), 409 (20.6), 391 (10.1), 330 (15.7), 315 (19.8), 297 (7.5), 285 (13.3), 271 (24.4), 269 (26.5), 255 (59.3), 234 (27.8), 229 (33.7), 218 (52.4), 203 (55.3), 189 (52.9), 175 (40.7), 159 (40.4), 145 (42.7), 133 (56.6), 119 (66.7), 109 (63.3), 95 (100.0), 81 (80.7), 69 (99.3), 55 (97.5), 43 (56.2); HRMS calcd. for C30H50O2: 442.3813; found: 442.3845.

Polpunonic acid (55):

Off-white needles (EtOH-H2O); mp 246-248°C (subl.); [α]D 25 -60.0° (c 0.60, CHCl3), IR (CHCl3) cm⁻¹ 2944 (CH), 1740 (C=O), 1702 (C=O), 1525, 1467, 1390, 1225, 1208, 929, 853, 790, 720, 675; 1H NMR (CDCl3) δ 0.73 (3H, s, CH3), 0.87 (3H, d, C4 CH3, J = 7 Hz), 0.87 (3H, s, CH3), 0.88 (3H, s, CH3), 1.00 (3H, s, CH3), 1.09 (3H, s, CH3), 1.27 (3H, s, CH3), 1.28 - 1.77 (m, aliphatic H), 1.95 - 2.43 (3H, m, C2 CH2, H4); MS m/z (rel. intensity) 456 (5.9), 441 (2.6), 438 (2.7), 423 (2.1), 410 (7.2), 395 (3.9), 371 (2.7), 342 (8.0), 327 (2.6), 303 (2.3), 287 (2.1), 273 (31.5), 250 (11.7), 246 (11.1), 231 (20.1), 218 (11.3), 204 (9.2), 189 (19.3), 175 (14.7), 163 (35.0), 155 (38.3), 147 (19.2), 137 (30.7), 121 (39.6), 109 (100.0), 95 (63.7), 81 (44.4), 69 (28.1), 55 (39.3); HRMS calcd. for C30H48O3: 456.3605; found: 456.3583.

3.6.5 Chromatographic separation of the spent medium extract metabolites

The spent medium extract (3.06 g) was partially separated by VLC using a 150 mL sintered glass funnel (medium frit, Merck silica 60G, 90 g). Stepwise elution was carried out using i) benzene (5×30 mL); ii) benzene-acetone 5:1 (19×30 mL); iii) benzene-acetone 4:1 (19×30 mL); iv) benzene-acetone 3:1 (12×30 mL); v) benzene-
acetone 2:1 (2×30 mL); vi) benzene-acetone 1:1 (18×30 mL) and ethyl acetate (250 mL) to give 22 crude fractions. All the fractions were complex mixtures of metabolites. Fractions 3, 4, 5, 6 and 7 (204.68 mg, 117.77 mg, 158.08 mg, 72.62 mg and 58.75 mg, respectively) were separated further by preparative TLC (Merck silica F254, 4×2 mm plates, fractions 6 and 7 were combined) using toluene-ethyl acetate-chloroform-formic acid 35:15:16:1 (the plates were eluted 3 times) to give oleanolic acid (127, 59.66 mg), the hydroxy acid, 160 (134.63 mg), triptolide (2, 179.09 mg) and a complex mixture (168.31 mg) which was not further investigated. Fractions 8, 9, 10, 11, 12 and 13 (71.00 mg, 113.66 mg, 44.34 mg, 84.0 mg, 63.94 mg and 222.09 mg, respectively) were separated by preparative TLC (Merck silica F254, 5×2 mm plates, 1×0.5 mm plate (fraction 10), toluene-ethyl acetate-chloroform-formic acid 35:15:16:1, the plates were eluted 4 times) to give 137 (13.22 mg), a mixture of 137 and 138 (10.30 mg), 138 (21.72 mg), a mixture of 138, 139 and 51 (42.48 mg), 139 (8.73 mg), 51 (25.53 mg), tripdiolide (1, 199.77 mg) and a complex mixture (166.65 mg). Fractions 14, 15 and 16 (80.90 mg, 38.65 mg and 10.96 mg, respectively) were separated by preparative TLC (Merck silica F254, 1×2 mm plate (fraction 14), 1×0.5 mm plate (fractions 15 and 16), toluene-ethyl acetate-chloroform-formic acid 35:15:16:1, the plates were eluted 3 times) to give 51 (48.10 mg). Fractions 17 and 18 (56.27 mg and 151.71 mg respectively) were separated by preparative TLC (1×0.5 mm plate and 1×2 mm plate, respectively, benzene-methanol-acetic acid 90:5:5, the plates were eluted 3 times) to give a further quantity of 51 (30.67 mg). Attempted separation of fractions 19 to 22 (174.08 mg, 83.45 mg, 39.59 mg and 49.50 mg respectively) by preparative TLC (Merck silica F254, 2×2 mm plates, benzene-methanol-acetic acid 90:5:5, the plates were eluted 3 times) was unsuccessful due to the complexity of the mixture of polar compounds.

15-Hydroxy-18(4→3)abieta-3, 8, 11, 13-tetraen-18-oic acid (160):

Colourless needles (EtOH); mp 141-145°C; \([\alpha]^{22}_D +143.9\) (c 0.14, MeOH); UV (MeOH) \(\lambda_{max} 215\) (ε 1926); IR (CHCl₃) cm⁻¹ 3600 (OH), 2970 (CH), 2580 (OH, br),
1618 (C=C), 1500, 1439, 1410, 1375, 1268, 1119, 1061, 958, 900, 832; \( ^1 \)H NMR (CDCl\(_3\)) \( \delta \) 1.06 (3H, s, C10 CH\(_3\)), 1.58 (6H, s, C(CH\(_3\))\(_2\)), 2.14 (3H, brs, C4 CH\(_3\)), 1.59 - 2.66 (m, aliphatic H), 3.02 (2H, m, H7), 7.25 - 7.33 (3H, m, ArH); MS m/z (rel. intensity) 314 (16.6), 299 (38.7), 296 (66.7), 281 (50.6), 263 (8.6), 253 (7.4), 235 (24.8), 215 (23.9), 197 (80.2), 183 (47.9), 169 (19.9), 165 (25.5), 157 (54.9), 155 (27.6), 141 (28.9), 129 (34.8), 115 (28.3), 105 (31.2), 91 (27.1), 83 (29.1), 77 (26.0), 59 (34.1), 43 (100.0); HRMS calcd. for C\(_{20}\)H\(_{26}\)O\(_3\): 314.1883; found: 314.1880.

**Triptolide (2):**

Colourless needles (EtOH); mp 230-234°C; IR (CHCl\(_3\)) cm\(^{-1}\) 3510 (OH), 2950 (CH), 1750 (C=O), 1673 (C=C), 1440, 1400, 1344, 1210, 1072, 1033, 974, 918, 865; \( ^1 \)H NMR (CDCl\(_3\)) \( \delta \) 0.89 (3H, d, CH(CH\(_3\))\(_2\), J = 7 Hz), 1.02 (3H, d, CH(CH\(_3\))\(_2\), J = 7 Hz), 1.32 (3H, s, C10 CH\(_3\)), 2.12 - 2.73 (5H, m, H5, H6a, H15, C2 CH\(_2\)), 2.74 (1H, d, C14 OH, J = 15 Hz), 3.28 (1H, d, H7, J = 5 Hz), 3.43 (1H, d, H14, J = 10 Hz), 3.52 (1H, d, H12, J = 3 Hz), 3.89 (1H, d, H11, J = 3 Hz), 4.69 (2H, t, H19); MS m/z (rel. intensity) 360 (0.8), 342 (5.2), 327 (7.4), 313 (7.1), 299 (7.7), 285 (6.8), 271 (7.0), 193 (11.5), 179 (16.1), 165 (20.8), 151 (28.0), 135 (20.1), 121 (23.9), 105 (26.7), 97 (28.3), 91 (43.2), 77 (30.6), 71 (57.7), 55 (34.2), 43 (100.0); HRMS calcd. for C\(_{20}\)H\(_{24}\)O\(_6\): 360.1573; found: 360.1580. The above spectral data were found to be identical with those of an authentic sample of triptolide.

**Tripdiolide (1):**

Colourless plates (EtOH); mp 224-226°C (dec.); IR (CHCl\(_3\)) cm\(^{-1}\) 3580 (OH), 3530 (OH), 2910 (CH), 1759 (C=O), 1680 (C=C), 1449, 1415, 1350, 1230, 1079, 1032, 982, 921, 878, 720; \( ^1 \)H NMR (CDCl\(_3\)) \( \delta \) 0.89 (3H, d, CH(CH\(_3\))\(_2\), J = 7 Hz), 1.02 (3H, d, CH(CH\(_3\))\(_2\), J = 7 Hz), 1.33 (3H, s, C10 CH\(_3\)), 1.35 (1H, dd, H1\(\alpha\), J = 5, 14 Hz), 1.87 (1H, d, H1\(\beta\), J = 14 Hz), 2.10 (1H, t, H6\(\beta\), J = 12 Hz), 2.23 (2H, m, H6\(\alpha\), H15), 2.36 (1H, d, C2
OH, $J = 3$ Hz), 2.65 (1H, dd, H5, $J = 7$, 12 Hz), 2.75 (1H, d, C14 OH, $J = 10$ Hz), 3.52 (1H, d, H12, $J = 3$ Hz), 3.93 (1H, d, H11, $J = 3$ Hz), 4.63 (1H, br, H2α, $W_2^J = 10$ Hz), 4.78 (2H, t, H19); MS m/z (rel. intensity) 376 (0.3), 358 (16.0), 329 (4.2), 315 (5.2), 311 (5.5), 297 (7.6), 269 (12.3), 257 (9.7), 239 (17.3), 227 (13.1), 213 (10.8), 199 (12.6), 185 (10.4), 175 (12.9), 165 (13.9), 147 (23.8), 139 (17.5), 115 (20.4), 105 (18.0), 97 (22.9), 91 (29.9), 83 (18.2), 77 (25.1), 71 (43.1), 55 (32.2), 43 (100.0); HRMS calcd. for $C_{20}H_{24}O_7$: 376.1522; found: 376.1518. The above spectral data were found to be identical with those of an authentic sample of tripdiolide.
3.7 Synthesis of the allylic alcohol precursor, **171**

**Oxidative decarboxylation of dehydroabietic acid (80):**

Lead tetraacetate (75.30 g, 0.17 mol.) was added to a stirred solution of dehydroabietic acid (80, 45.00 g, 0.15 mol) in benzene (220 mL) and pyridine (18.1 mL) and the reaction mixture was stirred under nitrogen at room temperature for 1 hour and refluxed for 3 hours. The reaction mixture was cooled to room temperature, filtered through celite and the celite was washed with benzene. The filtrate and the washings were combined and the solvent was evaporated to yield a dark brown oil. The crude oil was passed through an alumina column (Brockman act. I, 80 - 200 Å, acid, 2 kg) using petroleum ether (35 - 60) to give 19.60 g (50.5%) of the olefin mixture, **175** as a colourless oil.

**Hydroboration-oxidation of the 18-norabietatetraenes, 175:**

Lithium aluminium hydride (4.50 g, 0.12 mol) was added gradually to a stirred solution of the 18-norabietatetraene mixture (175, 11.00 g, 0.04 mol) in anhydrous ether (220 mL) under nitrogen at 0°C. Boron trifluoride etherate (17.0 mL, 0.14 mol) in anhydrous ether (70 mL) was then added dropwise at the same temperature over 30 minutes. The reaction mixture was stirred at room temperature for 2.5 hours and cooled in a dry ice bath. Wet ether (50 mL) followed by ice and brine (100 mL) were added slowly

1See below for the spectral data of the separated olefins 188, 189 and 81.
to the reaction mixture and the organic layer was decanted. The solvent was evaporated and the residue was dissolved in tetrahydrofuran (270 mL) and 10% sodium hydroxide (204 mL). 30% Hydrogen peroxide (136 mL) was added and the reaction mixture was stirred at room temperature for 16 hours. The reaction mixture was extracted with ethyl acetate (2×100 mL) and the organic layers were combined, washed with water (2×45 mL) and dried over sodium sulphate (anhydrous). The solvent was evaporated to give 10.10 g of the norabietatrienols, 176, as a colourless oil.

The alcohol mixture (8.00 g, 0.03 mol) was separated by preparative HPLC (Waters Prep-pack 500 silica gel column, hexanes-ethyl acetate 5:1, flow rate 25 mL/min.). 200 mL fractions were collected and the solvent was evaporated to give 0.88 g (11.0%) of 19-norabieta-8, 11, 13-trien-3β-ol, 177, as a colourless oil; 2.80 g (35.0%) of 18-norabieta-8, 11, 13-trien-3α-ol, 178, as a white solid and 2.50 g (31.3%) of 18-norabieta-8, 11, 13-trien-19-ol, 179, as a colourless oil.

19-Norabieta-8, 11, 13-trien-3β-ol (177):

IR (CHCl₃) cm⁻¹ 3610 (OH, sharp), 3450 (OH, br), 2950 (CH), 1500 (Ar); ¹H NMR (CDCl₃) δ 1.15 (3H, d, C₄ CH₃, J = 8 Hz), 1.24 (6H, d, CH(CH₃)₂), 1.48 (3H, s, C₁₀ CH₃), 1.23 - 1.65 (m, aliphatic H), 2.21 (1H, m, H₄β), 2.84 (2H, m, H₇β, H₁₅), 3.13 (1H, dd, H₇α, J = 5, 16 Hz), 4.32 (1H, q, H₃α, J = 6 Hz), 6.94 (1H, brs, H₁₄), 7.05 (1H, brd, H₁₀), 7.23 (1H, d, H₁₁); MS m/z (rel. intensity) 272 (32.1), 257 (66.9), 254 (25.3), 243 (20.9), 239 (100), 201 (49.4), 197 (93.7), 187 (36.1), 183 (26.7), 169 (32.0), 159 (81.0), 157 (27.8), 155 (33.2), 153 (20.0), 145 (27.2), 143 (32.8), 141 (74.3); HRMS calcd. for C₁₉H₂₈O: 272.2141; found: 272.2141.

18-Norabieta-8, 11, 13-trien-3α-ol (178):

mp 110-114°C; IR (CHCl₃) cm⁻¹ 3620 (OH), 2950 (CH), 1500 (Ar); ¹H NMR (CDCl₃) δ 1.01 (3H, d, C₄ CH₃, J = 8 Hz), 1.17 (3H, s, C₁₀ CH₃), 1.23 (6H, d,
CH(CH$_{3}$)$_2$, J = 7 Hz), 1.30 - 2.15 (m, aliphatic H), 2.82 (1H, septet, H15, J = 7 Hz), 2.90 (2H, m, H7), 3.85 (1H, brs, H3$\beta$), 6.89 (1H, brs, H14), 6.99 (1H, brd, H12), 7.16 (1H, d, H11); MS m/z (rel. intensity) 272 (15.8), 257 (18.9), 239 (100.0); HRMS calcd. for C$_{19}$H$_{28}$O: 272.2141; found: 272.2138; Anal. calcd. for C$_{19}$H$_{28}$O: C 83.77, H 10.36; found: C 83.89, H 10.29.

18-Norabieta-8, 11, 13-trien-19-ol (179):

IR (neat) cm$^{-1}$ 3350 (OH), 2900 (CH), 1500 (Ar); $^1$H NMR (CDCl$_3$) $\delta$ 1.07 (3H, s, C10 CH$_3$), 1.26 (6H, d, CH(CH$_3$)$_2$, J = 7 Hz), 1.40 - 2.02 (m, aliphatic H) 2.92 (2H, m, H7), 3.76 (2H, m, CH$_2$OH), 6.90 (1H, brs, H14), 7.01 (1H, brd, H12), 7.19 (1H, d, H11); MS m/z (rel. intensity) 272 (39.5), 257 (100.0), 239 (78.8), 197 (68.1), 185 (30.3), 183 (21.8), 171 (21.5), 159 (73.2), 155 (29.1), 143 (33.1), 141 (73.5), 129 (45.4), 117 (50.0); HRMS calcd. for C$_{19}$H$_{28}$O: 272.2141; found: 272.2140.

18-Norabieta-8, 11, 13-trien-3-one (180):

Chromium trioxide-pyridine (Collins reagent, 30.60 g, 0.12 mol) was added to a stirred solution of the alcohol, 178, (2.69 g, 9.88 mmol) in methylene chloride (255 mL) and the reaction mixture was stirred at room temperature for 6 hours. The reaction mixture was filtered through florisil (30 mL) and the florisil was washed with ether. The filtrate and the washings were combined and the solvent was evaporated to give a crude yellow oil. Flash chromatography (Merck silica 60G, 100 g, hexanes-ethyl acetate 19:1) yielded 1.50 g (56.2%) of 180 as a white solid which was recrystallized from ether-petroleum ether (35-60). mp 68-69°C; IR (CHCl$_3$) cm$^{-1}$ 2900 (CH), 1685 (C=O); $^1$H NMR (CDCl$_3$) $\delta$ 1.21 (3H, d, C4 CH$_3$, J = 6 Hz), 1.23 (6H, d, CH(CH$_3$)$_2$, J = 7 Hz), 1.34 (3H, s, C10 CH$_3$), 1.59 (1H, m, H2o). 1.84 - 2.04 (2H, m, 1H), 2.19 (1H, dq, H4$\alpha$, J = 2, 6 Hz), 2.42 - 2.62 (3H, m, H5$\alpha$, H6), 2.75 (1H, ddd, H2$\beta$, J = 3, 7, 12 Hz), 2.84 (1H, septet, H15, J = 7 Hz), 2.93 (2H, m, H7), 6.92 (1H, brs, H14), 7.03 (1H, brd, H12), 7.19 (1H, d,
18-Norabieta-8, 11, 13-trien-3-p-toluenesulphonylhydrazone (181):

*p*-Toluenesulphonylhydrazide (1.15 g, 6.21 mmol) was added to a stirred solution of ketone, 180 (1.40 g, 5.18 mmol), in benzene (100 mL) at room temperature and under argon. Boron trifluoride etherate (175 µL) was added and the reaction was stirred at room temperature for 40 hours. The benzene was evaporated and the yellow residue was dissolved in ether (100 mL). The organic layer was washed with water (3 × 20 mL) and dried over Mg SO₄ (anhydrous). The solvent was evaporated and the yellow residue was subjected to flash chromatography (Merck silica 230-400 Å, 50 g, hexanes-ethyl acetate 3:1) to give 2.24 g (98.6%) of 181 as a light yellow solid. mp 70-72°C; IR (CHCl₃) cm⁻¹ 3210 (NH), 2950 (CH), 1700 (C=O), 1335 (SO₂NH), 1168 (SO₂NH); ¹H NMR (CDCl₃) δ 1.09 (3H, d, C₄ CH₃, J = 7 Hz), 1.15 (3H, s, C₁₀ CH₃), 1.21 (6H, d, CH(CH₃)₂, J = 7 Hz), 2.30 - 2.45 (m, aliphatic H), 2.23 (1H, dq, H₄β, J = 7, 11 Hz), 2.40 (3H, s, Ar CH₃), 2.76 - 2.90 (3H, m, H₇, H₁₅), 6.89 (1H, brs, H₁₄), 6.97 (1H brd, H₁₂), 7.14 (1H, d, H₁₁), 7.27 (2H, d, tosyl H₃, J = 8 Hz), 7.82 (2H, d, tosyl H₂, J = 8 Hz); MS m/z (rel. intensity) 438 (0.8), 424 (0.4), 283 (8.3), 254 (34.0), 239 (67.1), 225 (21.0), 199 (16.6), 186 (47.4), 91 (100.0); HRMS calcd. for C₂₆H₃₄N₂O₂S: 438.2341; found: 438.2341.

19 (4→3)abeo-abieta-2, 8, 11, 13-tetraen-19-ol (171):

*n*-Butyllithium (1.60 M, 18.0 mL, 30.00 mmol) was added to a stirred solution of 181 (1.30 g, 2.96 mmol) in tetramethylethylenediamine (TMEDA, 20 mL) at -72°C under
argon and the reaction mixture was stirred at -72°C for 15 minutes and at 0°C for 3 hours. Paraformaldehyde (1.50 g) was added and the reaction mixture was stirred at 0°C for 30 minutes and at room temperature for 18 hours. The solvent was evaporated and water (40 mL) was added. The solution was adjusted to pH 2 with 2N HCl and the precipitate was extracted into ether (3x40 mL). The organic layer was washed with 5% sodium bicarbonate (15 mL), water (15 mL) and dried over sodium sulphate (anhydrous). The solvent was evaporated and the crude yellow oil was chromatographed (Merck silica 230-400 Å, 50 g) by stepwise elution using: i) hexanes; ii) hexanes-ethyl acetate 9:1; and iii) hexanes-ethyl acetate 4:1 to give 460.00 mg (54.6%) of 171 as a colourless oil. IR (CHCl₃) cm⁻¹ 3600 (OH), 2950 (CH); ¹H NMR (CDCl₃) δ 1.10 (3H, s, C₁₀ CH₃), 1.15 (3H, d, C₄ CH₃, J = 7 Hz), 1.24 (6H, d, CH(CH₃)₂), 1.37 - 2.62 (m, aliphatic H), 2.80 - 2.93 (3H, m, H₇, H₁₅), 4.09 (1H, br, d, H₁₉, J = 12 Hz), 4.24 (1H, br, d, H₁₉, J = 12 Hz), 5.77 (1H, br, d, H₂), 6.91 (1H, brs, H₁₄), 7.02 (1H, br, d, H₁₂), 7.20 (1H, d, H₁₁); MS m/z (rel. intensity) 284 (25.2), 269 (14.2), 251 (8.7), 237 (3.6), 209 (12.4), 186 (100.0), 171 (38.6), 159 (22.7), 143 (27.1), 129 (24.7); HRMS calcd. for C₂₀H₂₈O: 284.2141; found: 284.2140; Anal. calcd. for C₂₀H₂₈O: C 84.45, H 9.92; found: C 84.66, H 10.07.

[19⁻¹⁴C] (4→3)abeo-abiesta-2, 8, 11, 13-tetraen-19-ol (182):

n-Butyllithium (1.60 M, 4.0 mL, 6.40 mmol) was added to a stirred solution of 181 (400.00 mg, 0.91 mmol) in TMEDA (7.0 mL) at -72°C under argon. The reaction mixture was stirred at -72°C for 15 minutes and at 0°C for 3 hours. ¹⁴C-paraformaldehyde (500 µCi, 1.11 x 10⁹ DPM) was added and the reaction mixture was stirred at 0°C for 1 hour. Inactive paraformaldehyde (424.00 mg) was added and the reaction was stirred for a further 18 hours at room temperature. Work-up and column chromatography were carried out as above for 171 to give 102.97 mg (39.7%) of 182 as a colourless oil. Activity 4.68 x 10⁶ DPM (0.4% incorporation); specific activity 5.82 mCi mol⁻¹ (1.29 x 10¹⁰ DPM mol⁻¹).
3.8 Biotransformation studies of the allylic alcohol, 171 and the radioactive alcohol, 182

Tissue culture cells of the TRP-4a cell line were grown in PRD$_2$Co$_{100}$ medium and resuspended in MSNA$_{0.5}$K$_{0.5}$ production medium according to the procedure outlined in Part A. The cells were incubated until the end of the growth phase (26 days, RI 1.3336), at which time, the allylic alcohol 171, and the radioactive alcohol 182, were added. The radioactive alcohol, 182 (100.00 mg, 4.59x10$^6$ DPM) in methanol (4.0 mL) was added to 1 L of suspension culture and the inactive alcohol, 171 (500.00 mg) in methanol (7.0 mL) was added to to 5 L of suspension culture. The suspension cultures were incubated for a further 120 hours and harvested. The cells and the spent medium were separated by filtration and frozen prior to extraction.

**Extraction of the culture incubated with the radioactive alcohol, 182:**

**Cells:** The cells (wet wt. 156.9 g) were homogenized in a Waring blender with ethyl acetate (200 mL) and filtered through celite. The cell residue was washed with ethyl acetate (100 mL) and the combined filtrate and washings were transferred to a separatory funnel. The aqueous layer was separated, extracted with ethyl acetate (3x100 mL) and discarded. The cell residue was further extracted by sonication in methanol (200 mL) at 0°C for 2 hours, followed by filtration through celite and washing with methanol. The organic phases were combined and the solvent was evaporated to give a crude brown extract (3.68 g, 3.70x10$^6$ DPM, 80.6%).

**Spent medium:** The spent medium (892 mL) was saturated with sodium chloride and extracted with ethyl acetate (3x300 mL). The organic layers were combined and the solvent was evaporated to give a crude brown extract (174.1 mg, 7.35x10$^5$ DPM, 16.0%). Total activity recovered from the culture: 4.44x10$^6$ DPM (96.6%).
Extraction of the culture incubated with 171:

**Cells:** The cells (744.1 g) were extracted as above with ethyl acetate (600 mL and 3×200 mL) and methanol (600 mL) to give a crude brown extract (5.48 g).

**Spent medium:** The spent medium (4.30 L) was extracted as above with ethyl acetate (4×700 mL) to give a crude brown extract (281.30 mg).

Radioactivity profiles of the radioactive spent medium extract:

The spent medium extract (5.86 mg, 3.42×10^4 DPM) was applied to an analytical TLC plate (Merck silica F₃₅₄, 20×20 cm, 0.25 mm with concentrating zone) and eluted with hexane-ethyl acetate 9:1. A small strip of the plate was cut and visualized using anisaldehyde-H₂SO₄. Bands were cut according to the positions of the visualized spots and the silica gel from each band was scraped directly into scintillation vials. Methanol (0.5 mL), water (0.5 mL) and liquid scintillation cocktail (10 mL) were added to each vial and the vials were sonicated for 10 minutes and counted. The results are presented in Table 11 and Figure 22.

The procedure was repeated using 5.30 mg (3.09×10^4 DPM) of the spent medium extract and eluted with chloroform-methanol-acetic acid 50:5:1 (the plate was eluted twice). The results are presented in Table 12 and Figure 23.

Radioactivity profiles of the radioactive cell extract:

A portion of the radioactive cell extract (1.39 g, 1.40×10^6 DPM) was filtered through silica gel using ethyl acetate, to separate the polar material from the non-polar material, to give fraction 1 (60.25 mg, 7.79×10^5 DPM) as the non-polar material, and the column was flushed with methanol to give fraction 2 (1.00 g, 3.09×10^5 DPM) as the polar material.
Radioactivity profiles were carried out as above on fraction 1 (4.09 mg, 5.29×10^4 DPM, hexanes-ethyl acetate 9:1, and 3.87 mg, 4.99×10^4 DPM, chloroform-methanol-acetic acid 95:5:1). The results are presented in Tables 13 and 14 respectively and Figures 24 and 25 respectively. A radioactivity profile was also carried out on fraction 2 (26.59 mg, 8.19×10^3 DPM, chloroform-methanol-acetic acid 95:5:1). The results are presented in Table 15 and Figure 26.

**Isolation of the radioactive metabolites from the radioactive extracts**

The radioactive extracts were combined (3.80×10^6 DPM), suspended in water (50 mL) and extracted into ethyl acetate (3×75 mL). The organic layers were combined, washed with water (10 mL) and dried over sodium sulphate (anhydrous). The solvent was evaporated to give a crude brown residue (309.70 mg, 3.42×10^6 DPM). Column chromatography of the residue (Merck silica 230-400 Å, 40 g) was carried out using chloroform-methanol-acetic acid 95:5:1 and the column was flushed with methanol to remove the polar material. Seven crude fractions were collected. Fraction 1 was an oily fraction containing fatty acid-like compounds (13.21 mg, 1.50×10^4 DPM). Fraction 2 (61.85 mg, 1.00×10^6 DPM) contained the aldehyde, 183, while fraction 3 (50.83 mg, 8.06×10^5 DPM) contained the starting material, 182 and the acid, 184. Fractions 4, 5 and 6 (21.53 mg, 1.85×10^5 DPM; 25.77 mg, 1.82×10^5 DPM and 14.28 mg, 8.06×10^4 DPM respectively) contained further quantities of the acid, 184. Rapid elution with methanol yielded fraction 7 (174.46 mg, 2.69×10^5 DPM) containing trioloxide (1).

**Isolation of the radioactive aldehyde, 183**

Fraction 2 (61.85 mg, 1.00×10^6 DPM) was separated by preparative TLC (Merck silica F254, 0.5 mm) using hexanes-ethyl acetate 19:1 (the plate was eluted twice) to give the radioactive aldehyde, 183 (19.86 mg, 8.13×10^5 DPM) as a colourless oil.
Isolation of the radioactive alcohol, 182, and the radioactive acid, 184:

Fractions 3 and 4 were combined (72.36 mg, $9.92 \times 10^5$ DPM) and separated by preparative TLC (Merck silica F$_{254}$, 0.5 mm, 2 plates) using chloroform-methanol-acetic acid 95:5:1 to give the allylic alcohol, 182 (13.04 mg, $1.54 \times 10^5$ DPM) and the crude acid, 184 (40.64 mg, $8.04 \times 10^5$ DPM). The crude acid was combined with fractions 5 and 6 (80.69 mg, $1.07 \times 10^6$ DPM) and separated by preparative TLC (Merck silica F$_{254}$, 0.5 mm, 2 plates, hexanes-ethanol-acetic acid 95:5:1, the plates were eluted 4 times) to give the radioactive acid, 184 (40.64 mg, $8.04 \times 10^5$ DPM), as a white solid.

Isolation of tripdiolide (1) from fraction 7:

Fraction 7 (174.46 mg, $2.69 \times 10^5$ DPM) was separated by preparative TLC (Merck silica F$_{254}$, 2 mm) using chloroform-methanol-acetic acid 95:5:1 (the plate was eluted 3 times) to give crude tripdiolide (1, 6.60 mg, $2.77 \times 10^4$ DPM). A further separation by preparative TLC (Merck silica F$_{254}$, 0.5 mm) using chloroform-methanol-acetic acid 95:5:1 (the plate was eluted twice) gave tripdiolide (1, 2.02 mg, $6.92 \times 10^3$ DPM).

Isolation of the aldehyde, 185, alcohol, 171, and acid, 186, from the inactive cell extract:

The inactive cell extract (5.48 g) was suspended in water (50 mL) and extracted into ethyl acetate (3×100 mL). The organic layers were combined, washed with water (30 mL) and dried over sodium sulphate (anhydrous). The solvent was evaporated to give 1.09 g of a crude brown extract. The crude extract was separated by column chromatography (Merck silica 230-400 Å, 120 g) using gradient elution from hexanes to ethyl acetate and rapid elution of the column with methanol to remove the polar material. Three crude fractions were obtained. Fraction 1 (343.27 mg) contained the aldehyde, 185 and the alcohol, 171, and fraction 2 (218.66 mg) contained the acid, 186. Fraction 3 (296.24 mg) contained the polar material flushed from the column with methanol.
Isolation of the aldehyde, 185 and the alcohol 171, from fraction 1:

Fraction 1 (343.27 mg) was separated by preparative TLC (Merck silica F254, 2 mm, 2 plates) using chloroform-methanol-acetic acid 95:5:1 to give the crude aldehyde, 185 (139.55 mg) and the crude alcohol, 171 (168.51 mg). The crude aldehyde was purified further by preparative TLC (Merck silica F254, 2 mm) using hexanes-ethyl acetate 9:1 to give the aldehyde, 185 (38.54 mg), as a colourless oil. Its $^1$H NMR, MS, TLC and IR were identical with those of the synthetic aldehyde (vide infra). IR (neat) cm$^{-1}$ 2975 (CH), 1695 (C=O); $^1$H NMR (CDCl$_3$) $\delta$ 1.03 (3H, s, C10 CH$_3$), 1.24 (6H, d, CH(CH$_3$)$_2$), 1.25 (3H, d, C4 CH$_3$), 1.38 - 2.93 (m, aliphatic H), 6.83 (1H, brd, H2), 6.94 (1H, brs, H14), 7.04 (1H, brd, H12), 7.21 (1H, d, H11), 9.46 (1H, s, CHO); MS m/z (rel. intensity) 282 (17.0), 267 (21.4), 239 (5.9), 186 (100.0), 171 (47.3), 159 (38.8), 152 (6.5), 143 (41.6), 128 (48.8), 115 (42.1).

The crude alcohol, 171, was purified further by preparative TLC (Merck silica F254, 2 mm, 2 plates) using chloroform-methanol 98:2 to give pure alcohol (24.85 mg) and crude alcohol (49.57 mg). The crude fraction was purified further by preparative TLC (Merck silica F254, 0.5 mm) using methylene chloride-methanol-acetic acid 100:6:1 to give a further quantity of the alcohol, 171 (3.84 mg), as a colourless oil. Its $^1$H NMR, MS, TLC and IR were identical with those of the starting material.

Isolation of the acid, 186, from fraction 2:

Fraction 2 (218.66 mg) was separated by preparative TLC (Merck silica F254, 2 mm, chloroform-methanol-acetic acid 95:5:1) to give the acid, 186 (96.59 mg), as a white solid and 41.45 mg of crude acid which was separated again by the same procedure to give a further quantity of the acid (26.64 mg) as a white solid. Its $^1$H NMR, MS, TLC, mp and IR were identical with those of the synthetic material (vide infra). mp 153-158°C; IR
(CHCl₃) cm⁻¹ 2975 (CH), 1700 (C=O); ¹H NMR (CDCl₃) δ 1.08 (3H, s, C₁₀ CH₃), 1.25 (6H, d, CH(CH₃)₂), 1.26 (3H, d, C₄ CH₃), 1.33 - 2.95 (m, aliphatic H), 6.94 (1H, brs, H1₄), 7.03 (1H, brd, H1₂), 7.07 (1H, brd, H₂), 7.19 (1H, d, H1₁); MS m/z (rel. intensity) 298 (18.8), 283 (26.0), 269 (3.6), 256 (4.1), 239 (6.4), 223 (4.5), 209 (5.5), 195 (10.2), 186 (100.0), 171 (29.9), 159 (50.9), 143 (22.9), 129 (25.4), 117 (19.3).

19(4→3)abeo-abieta-2, 8, 11, 13-tetraen-19-al (185):

Pyridinium chlorochromate (227.00 mg, 1.05 mmol) was added to a stirred solution of the alcohol, 171 (50.00 mg, 0.18 mmol) in methylene chloride (5 mL) and the reaction mixture was stirred at room temperature for 1.5 hours. The reaction mixture was filtered through florisil (10 mL) and the florisil was washed with ether. The filtrate and the washings were combined and the solvent was evaporated to give a yellow oil. The crude product was purified by preparative TLC (Merck silica F₂₅₄, 2 mm) using hexanes-ethyl acetate 19:1 (the plate was eluted twice) to give 17.60 mg (35.5%) of 185 as a colourless oil. IR (neat) cm⁻¹ 2975 (CH), 1695 (C=O); ¹H NMR (CDCl₃) δ 1.03 (3H, s, C₁₀ CH₃), 1.24 (6H, d, CH(CH₃)₂), 1.25 (3H, d, C₄ CH₃), 1.38 - 2.93 (m, aliphatic H), 6.83 (1H, brd, H₂), 6.94 (1H, brs, H1₄), 7.04 (1H, brd, H1₂), 7.21 (1H, d, H1₁), 9.46 (1H, s, CHO); MS m/z (rel. intensity) 282 (17.0), 267 (21.4), 239 (5.9), 186 (100.0), 171 (47.3), 159 (38.8), 152 (6.5), 143 (41.6), 128 (48.8), 115 (42.1); HRMS calcd. for C₂₀H₂₆O: 282.1984; found: 282.1982.

19(4→3)abeo-abieta-2, 8, 11, 13-tetraen-19-oic acid (186):

Jones reagent was added dropwise to a stirred solution of the alcohol, 171 (45.00 mg, 0.16 mmol) in acetone (10 mL) until the orange colour persisted and the reaction mixture was stirred at room temperature for 3 hours. The reaction mixture was adjusted to pH 10 by addition of 10% NaOH and the solution was filtered to remove the green
precipitate. The solution was acidified to pH 2 using 5% HCl and the acetone was evaporated. The residual solution was extracted with ethyl acetate (2×5 mL) and the organic layers were combined, washed with water (2×2 mL) and dried over sodium sulphate (anhydrous). The solvent was evaporated to give a crude colourless oil which was purified by preparative TLC (Merck silica F$\text{2}_{54}$, 2 mm) using chloroform-methanol (9:1) to give 3.36 mg (7.1%) of the acid, 186, as a white solid. mp 155-158°C; IR (CHCl$_3$) cm$^{-1}$ 2975 (CH), 1700 (C=O); $^1$H NMR (CDCl$_3$) $\delta$ 1.08 (3H, s, C$_3$O CH$_3$), 1.25 (6H, d, CH(CH$_3$)$_2$), 1.26 (3H, d, C$_4$ CH$_3$), 1.33 - 2.95 (m, aliphatic H), 6.94 (1H, brs, H$_{14}$), 7.03 (1H, brd, H$_{12}$), 7.07 (1H, brd, H$_2$), 7.19 (1H, d, H$_{11}$); MS m/z (rel. intensity) 298 (18.8), 283 (26.0), 269 (3.6), 256 (4.1), 239 (6.4), 223 (4.5), 209 (5.5), 195 (10.2), 186 (100.0), 171 (29.9), 159 (50.9), 143 (22.9), 129 (25.4), 117 (19.3); HRMS calcd. for C$_{20}$H$_{26}$O$_2$: 298.1932; found:298.1932.

Radioactive 2, 4-dinitrophenylhydrazone derivative, 187, of the aldehyde, 183:

2, 4-Dinitrophenylhydrazine (15.50 mg, 0.08 mmol) was dissolved in methanol (0.5 mL) and concentrated H$_2$SO$_4$ (3 drops) and the solution was warmed to 50°C with stirring. A solution of the radioactive aldehyde, 183 (19.90 mg, 0.07 mmol, 8.13×10$^5$ DPM) in methanol (0.5 mL) was added and an orange precipitate formed immediately. The mixture was cooled to room temperature and centrifuged. The supernatant was removed and the residue was washed with methanol (2×1 mL). The orange residue was dried under vacuum and recrystallized from methylene chloride-hexanes to give 32.02 mg (7.62×10$^5$ DPM, 93.8%) of the dinitrophenylhydrazone, 187, as orange coloured crystals.

Recrystallization to constant activity of the 2, 4-dinitrophenylhydrazone, 187:

The 2, 4-Dinitrophenylhydrazone, 187 (32.02 mg, 7.62×10$^5$ DPM) was recrystallized from ether-methylene chloride, centrifuged and the supernatant was
removed. The crystals were dried and weighed and a sample was counted. Recrystallization was repeated until a constant specific activity was reached. The results are presented in Table 18.

**Recrystallization to constant activity of the radioactive acid, 184:**

The radioactive acid, 184 (16.87 mg, 7.24×10^5 DPM) was diluted with inactive acid, 186 (33.74 mg), isolated from the cell extract, to 50.61 mg and recrystallized from hexanes. Recrystallization to a constant specific activity was carried out according to the above procedure and the results are presented in Table 18.

**Recrystallization of tripdiolide (I) isolated from the radioactive spent medium extract:**

The tripdiolide fraction (2.02 mg, 6.92×10^3 DPM) was diluted to 7.22 mg with an authentic sample of tripdiolide (I) and recrystallized from ethanol. Recrystallization to a constant specific activity was carried out according to the above procedure and the results are presented in Table 18.

3.9 Synthetic studies towards isodehydroabietenolide (91)

**Separation of the 18-norabietatetraenes, 175:**

Silver nitrate (22.50 g) was dissolved in acetonitrile (375 mL) and the solution was added to silica gel (Merck silica 230-400 Å, 150 g). The slurry was stirred for 10 minutes and the solvent was evaporated. The silica gel was dried under vacuum, in the dark, overnight and at room temperature. The silver nitrate-silica gel was packed into a
column using hexanes and the olefin mixture, 175 (3.10 g, 0.01 mol) was applied. Gradient elution was carried out from hexanes to hexanes-ethyl acetate 98:2 to give 0.39 g (12.6%) of 18-norabieta-4, 8, 11, 13-tetraene, 188, 0.95 g (30.6%) of 18-norabieta-3, 8, 11, 13-tetraene, 189 and 1.76 g (56.8%) of 18-norabieta-4(19), 8, 11, 13-tetraene, 81.

18-Norabieta-4, 8, 11, 13-tetraene (188):

IR (neat) cm⁻¹ 2950 (CH), 1660 (C=C); ¹H NMR (CDCl₃) δ 1.23 (6H, d, CH(CH₃)₂), 1.38 (3H, s, C10 CH₃), 1.68 (3H, brs, C4 CH₃), 1.52 - 2.80 (m, aliphatic H), 6.89 (1H, brs, H14), 7.03 (1H, brd, H12), 7.20 (1H, d, H11); MS m/z (rel. intensity) 254 (6.07), 239 (100.0) 197 (9.0), 195 (9.3), 178 (4.9), 165 (11.6) 155 (10.0), 141 (14.2); Anal. calcd. for C₁₉H₂₆: C 89.70, H 10.30; found C 89.70, H 10.29.

18-Norabieta-3, 8, 11, 13-tetraene (189):

IR (neat) cm⁻¹ 2950 (CH), 1660 (C=C); ¹H NMR (CDCl₃) δ 1.04 (3H, s, C10 CH₃), 1.24 (6H, d, CH(CH₃)₂), 1.72 (3H, brs, C4 CH₃), 1.52 - 2.95 (m, aliphatic H), 5.44 (1H, brs, H3), 6.94 (1H, brs, H14), 7.00 (1H, brd, H12), 7.24 (1H, d, H11); MS m/z (rel. intensity) 254 (43.4), 239 (100.0), 225 (4.7), 211 (5.53), 199 (21.5), 197 (21.0), 183 (9.7), 171 (5.7), 169 (7.5), 165 (9.4), 159 (13.5), 141 (14.9); Anal. calcd. for C₁₉H₂₆: C 89.70, H 10.30; found: C 89.85, H 10.30.

18-Norabieta-4(19), 8, 11, 13-tetraene (81):

IR (neat) 3050 (C=CH₂), 2925 (CH), 1650 (C=C); ¹H NMR (CDCl₃) δ 1.02 (3H, s, C10 CH₃), 1.25 (6H, d, CH(CH₃)₂), 1.51 - 2.92 (m, aliphatic H), 4.61 (1H, d, H4, J = 2 Hz), 4.86 (1H, d, H4, J = 2 Hz), 6.94 (1H, brs, H14), 7.01 (1H, brd, H12), 7.22 (1H, d, H11); MS m/z (rel. intensity) 254 (42.9), 239 (92.9), 211 (4.5), 197 (100.0), 183 (4.7), 171 (2.8), 169 (9.1), 155 (11.9), 141 (14.5); Anal. calcd. for C₁₉H₂₆: C 89.70, H 10.30; found: C 89.90, H 10.22.
4α, 19α-Epoxv-18-norabieta-8, 11, 13-triene (190):

A solution of m-chloroperbenzoic acid (940.00 mg, 5.45 mmol) in methylene chloride (30 mL) was added gradually over 10 minutes to a stirred solution of the olefin, 81 (1.20 g, 4.72 mmol) in methylene chloride (10 mL) at 20°C. The reaction was stirred for 6 hours and the excess m-CPBA was destroyed by addition of 10% sodium sulphite until the starch-iodide paper test was negative. The organic layer was separated and washed with 5% sodium bicarbonate (3×15 mL), water (15 mL) and brine (15 mL) and dried over sodium sulphate (anhydrous). The solution was filtered through silica gel (15 mL) in a sintered glass funnel and the silica gel was washed with methylene chloride. The filtrate and the washings were combined and the solvent was evaporated. Column chromatography (Merck silica 230-400 Å, 100 g) using hexanes-ethyl acetate 19:1 gave 904.80 mg (70.9%) of 190 as a colourless oil, which solidified on standing at -30°C. mp 66-67.5°C; IR (neat) 3020 (CH-O), 2950 (CH); ¹H NMR (CDCl₃) δ 1.14 (3H, s, C10 CH₃), 1.23 (6H, d, CH(CH₃)₂), 1.28 - 2.32 (m, aliphatic H), 2.64 (1H, d, H19, J = 4 Hz), 2.77 - 3.05 (3H, m, H7, H19), 6.93 (1H, brs, H14), 7.01 (1H, brd, H12), 7.21 (1H, d, H11); MS m/z (rel. intensity) 270 (33.7), 255 (100.0), 239 (24.2), 237 (25.4), 227 (44.3), 225 (25.3), 211 (10.16), 197 (32.4), 195 (57.1), 185 (54.2), 171 (16.2), 167 (25.7), 165 (26.7), 159 (37.6), 155 (24.3), 153 (23.9), 143 (27.2), 141 (39.4), 129 (39.7), 117 (43.3); HRMS calcd. for C₁₉H₂₅O: 270.1984; found: 270.1986; Anal. calcd. for C₁₉H₂₅O: C 84.39, H 9.69; found: C 84.09, H 9.55.

18-Norabieta-3, 8, 11, 13-tetraen-19-ol (191):

n-Butyllithium (1.60 M, 8.36 mL, 13.38 mmol) was added to a stirred solution of diisopropylamine (1.96 mL, 14.05 mmol) in benzene (40 mL) at room temperature under argon. The reaction mixture was stirred at room temperature for 55 minutes and cooled to 0°C. Diethylaluminium chloride (7.44 mL, 13.38 mmol) was added and the reaction
mixture was stirred for a further 30 minutes at 0°C. A solution of the epoxide, 190 (904.80 mg, 3.35 mmol) in benzene (20 mL) was added to the reaction mixture and stirring was continued for 17 hours at room temperature. Water (15 mL) was added and the benzene was evaporated. The aqueous residue was acidified to pH 2 with 2N HCl and extracted with ether (3×50 mL). The organic layers were combined, washed with water (3×20 mL), dried over sodium sulphate (anhydrous) and the solvent was evaporated to give a yellow oil. Column chromatography (Merck silica 230-400 Å, 100 g) using hexanes-ethyl acetate 9:1 and hexanes-ethyl acetate 4:1 gave 549.60 mg (60.7%) of 191 as a colourless oil. IR (neat) cm⁻¹ 3300 (OH), 2925 (CH); ^1^H NMR (CDCl₃) δ 1.08 (3H, s, C₁₀ CH₃), 1.29 (6H, d, CH(CH₃)₂), 1.32 - 3.02 (m, aliphatic H), 4.11 (1H, d, H₁₉, J = 12 Hz), 4.23 (1H, d, H₁₉, J = 12 Hz), 5.78 (1H, brs, H₃), 6.98 (1H, brs, H₁₄), 7.03 (1H, brd, H₁₂), 7.27 (1H, d, H₁₁); MS m/z (rel. intensity) 270 (9.6), 255 (20.6), 253 (22.7), 239 (34.4), 237 (51.1), 225 (17.3), 211 (17.8), 209 (25.6), 195 (100.0), 181 (48.4), 178 (51.5), 165 (78.1), 153 (51.3), 141 (85.9), 129 (74.6), 115 (96.1).

3α, 4α-Epoxy-18-norabieta-8, 11, 13-triene (192):

A solution of m-chloroperbenzoic acid (800.00 mg, 4.64 mmol) in methylene chloride (20 mL) was added gradually over 15 minutes to a stirred solution of the olefin, 189 (650.00 mg, 2.56 mmol) in methylene chloride (10 mL) at room temperature. The reaction mixture was stirred for 2 hours and 10% sodium sulphite was added until the starch-iodide paper test was negative. The organic layer was separated and washed with 5% sodium bicarbonate (3×25mL), water (3×25 mL), brine (10 mL) and dried over sodium sulphate (anhydrous). The solvent was evaporated to give a colourless oil. Column chromatography (Merck silica 230-400 Å, 35 g) using hexanes-ethyl acetate 19:1 gave 464.30 mg (67.2%) of 192 as a white solid. mp 71-72°C; IR (CHCl₃) cm⁻¹ 3000 (CH-O), 2950 (OH); ^1^H NMR (CDCl₃) δ 1.07 (3H, s, C₁₀ CH₃), 1.22 (6H, d, CH(CH₃)₂), 1.34 (3H, s, C₄ CH₃), 1.39 - 2.20 (m, aliphatic H), 2.77 - 2.94 (3H, m, H₇, H₁₅), 3.01 (1H, brd,
H3, J = 2 Hz), 6.91 (1H, brs, H14), 6.98 (1H, brd, H12), 7.18 (1H, d, H11); MS m/z (rel. intensity) 270 (24.1), 255 (100.0), 239 (15.6), 237 (18.6), 225 (10.0), 211 (8.6), 195 (44.6); HRMS calcd. for C19H26O: 270.1985; found: 270.1988; Anal. calcd. for C19H26O: C 84.39, H 9.69: found: C 84.17, H 9.75.

18-Norabieta-4(19), 8, 11, 13-tetraen-3α-ol (82):

**Method A:** Acetic acid (0.1 mL), selenium dioxide (0.27 g, 2.43 mmol) and 90% t-butyl hydroperoxide (3.2 mL, 35.55 mmol) were added to a stirred solution of the olefin, 81 (3.30 g, 13.07 mmol) in methylene chloride (20 mL) at room temperature and the reaction mixture was stirred for 4 hours. Ether (100 mL) was added and the organic layer was washed with 10% NaOH (3×5 mL), water (3×10 mL) and brine (5 mL). The organic layer was dried over magnesium sulphate (anhydrous) and the solvent was evaporated to give a yellow residue. The residue was separated by VLC (Merck silica 60G, 60 g) and stepwise elution using: i) hexanes; ii) hexanes-ethyl acetate 9:1 and iii) hexanes-ethyl acetate 4:1 to give 2.42 g (68.4%) of 82 as a white solid.

**Method B:** n-Butyllithium (1.60 M, 1.50 mL, 2.40 mmol) was added to a stirred solution of diisopropylamine (243.00 mg, 2.40 mmol) in benzene (10 mL) under argon at 0°C. The reaction mixture was stirred for 1 hour at room temperature and cooled to 0°C. Diethylaluminium chloride (1.33 mL, 2.40 mmol) was added and the reaction mixture was stirred for a further 30 minutes at 0°C. A solution of the epoxide, 192 (162.30 mg, 0.60 mmol) was added at 0°C and the reaction mixture was stirred at room temperature for 18 hours. Water (5 mL) was added and the benzene was evaporated. The aqueous residue was acidified to pH 2 with 2N HCl and extracted with ether (3×20 mL). The organic layer was washed with water (3×5 mL), brine (5 mL) and dried over sodium sulphate (anhydrous). The solvent was evaporated to give a white solid which was filtered, using hexanes-ethyl acetate 4:1, through silica gel (Merck silica 230-400 Å, 30 mL) in a
sintered glass funnel to give 162.30 mg (100.0%) of 82 as a white solid. mp 60-61°C; IR (CHCl₃) cm⁻¹ 3608 (OH), 2962 (CH), 1650 (C=C); ¹H NMR (CDCl₃) δ 0.98 (3H, s, C10 CH₃), 1.23 (6H, d, CH(CH₃)₂), 1.41 (1H, brs, OH), 1.72 - 2.97 (m, aliphatic H), 4.38 (1H, brs, H3β, W½ = 6 Hz), 4.75 (1H, s, H19), 5.08 (1H, s, H19), 6.94 (1H, brs, H14), 7.02 (1H, brd, H12), 7.23 (1H, d, H11); MS m/z (rel. intensity) 270 (20.8), 255 (11.5), 237 (100.0), 195 (46.1), 185 (8.2), 167 (17.2), 153 (13.6), 141 (13.6), 128 (13.4), 115 (12.7); HRMS calcd. for C₁₉H₂₆O: 270.1985; found: 270.1984.

**19-Chloro-18-norabieta-3, 8, 11, 13-tetraene (193):**

A solution of the allylic alcohol, 82 (39.50 mg, 0.15 mol) in ether (1 mL) was added to a stirred solution of thionyl chloride (10.0 µL, 0.17 mmol) in ether (1 mL) and the reaction mixture was stirred at room temperature for 2 hours. The solvent was evaporated to give a colourless oil which was purified by preparative TLC (Merck silica F₂₅₄, 0.5 mm) using hexanes to give 34.90 mg (82.7%) of 193 as a colourless oil. IR (CHCl₃) cm⁻¹ 2950 (CH), 839 (C-Cl); ¹H NMR (CDCl₃) δ 1.03 (3H, s, C10 CH₃), 1.23 (6H, d, CH(CH₃)₂), 1.53 - 2.55 (m, aliphatic H), 2.84 (1H, septet, H15), 2.97 (2H, m, H7), 4.04 (1H, d, H19, J = 10 Hz), 4.21 (1H, d, H19, J = 10 Hz), 5.89 (1H, brs, H3), 6.97 (1H, brs, H14), 7.01 (1H, brd, H12), 7.24 (1H, d, H11); MS m/z (rel. intensity) 290 (0.2), 288 (1.0), 275 (0.4), 273 (2.3), 252 (47.8), 237 (65.1), 209 (25.8), 195 (100.0), 181 (16.7), 178 (38.7), 171 (30.9), 167 (44.5), 165 (50.7), 153 (27.6), 141 (32.3), 128 (39.6), 117 (27.5), 115 (41.8); HRMS calcd. for C₁₉H₂₅Cl: 288.1645; found: 288.1649.

**Bromination of the allylic alcohol, 82:**

**Method A:** Dimethyl sulphide (27.5 µL, 0.37 mmol) was added to a stirred solution of N-bromosuccinimide (65.80 mg, 0.37 mmol) in methylene chloride (2mL), under nitrogen at room temperature, to give a bright yellow solution. A solution of the
allylic alcohol, 82 (50.00 mg, 0.18 mmol) in methylene chloride (1 mL) was added at 0°C and the reaction mixture was stirred at 0°C for 2 hours. The reaction mixture was diluted with ether (20 mL) and washed with water (2×10 mL) and brine (5 mL). The organic layer was dried over sodium sulphate (anhydrous) and the solvent was evaporated to give a crude yellow oil. Preparative TLC separation (Merck silica F254, 0.5 mm) using hexanes-ethyl acetate 19:1 gave 25.77 mg (41.8%) of the 3β-bromide, 194 and the Δ3, 19-bromide, 195, as a yellow oil (1:1 mixture by 1H NMR), and 21.00 mg (26.5%) of the dibromo-3α-ol, 196, as a yellow oil.

**Method B**: Triphenylphosphine (21.70 mg, 0.08 mmol) and carbon tetrabromide (27.40 mg, 0.08 mmol) were added to a stirred solution of the allylic alcohol, 82 (20.30 mg, 0.08 mmol) in acetonitrile (2 mL) at room temperature and the reaction mixture was stirred for 24 hours. The solvent was evaporated and the residue was separated by preparative TLC (Merck silica F254, 0.5 mm) using hexanes-ethyl acetate 19:1 to give 6.00 mg (29.5%) of the starting material, 82, 7.90 mg (31.6%) of the allylic bromides, 194 and 195 and 0.8 mg (4.2%) of the olefin, 197 as a colourless oil.

3α-Bromo-18-norabieta-4(19), 8, 11, 13-tetraene (194) and 19-bromo-18-norabieta-3, 8, 11, 13-tetraene (195):

IR (neat) cm⁻¹ 2950 (CH), 1655 (C=C); ¹H NMR (CDCl₃) δ 0.98 (3H, s, C10 CH₃), 1.04 (3H, s, C10 CH₃), 1.23 (6H, d, CH(CH₃)₂), 1.24 (6H, d, CH(CH₃)₂), 1.51 - 3.05 (m, aliphatic H), 4.01 (1H, d, H19 (CH₂Br) J = 10 Hz), 4.16 (1H, d, H19 (CH₂Br), J = 10 Hz), 4.82 (1H, s, H19 (olefin H)), 5.09 (1H, dd, H3β (CHBr), J = 2, 4 Hz), 5.23 (1H, s, H19 (olefin H)), 5.94 (1H, brs, H3 (olefin H)), 6.94 and 6.96 (1H each, brs, H14), 7.01 and 7.03 (1H each, brd, H12), 7.23 and 7.24 (1H each, d, H11); MS m/z (rel. intensity) 334 (0.1), 332 (0.2), 319 (0.7), 317 (0.5), 252 (42.0), 237 (62.1), 209 (26.4), 195 (100.0), 178 (42.4), 165 (67.5), 152 (39.5), 141 (48.1), 128 (51.8), 115 (61.5); HRMS calcd. for C₁₉H₂₅Br: 332.1176; found: 332.1141.
4α, 19-Dibromo-18-norabiet-8, 11, 13-trien-3α-ol (196):

IR (CHCl₃) cm⁻¹ 3600 (OH), 2955 (CH); ¹H NMR (CDCl₃) δ 1.23 (6H, d, CH(CH₃)₂), 1.39 (3H, s, C10 CH₃), 1.87 - 2.48 (m, aliphatic H), 2.84 (1H, septet, H15), 2.99 (2H, m, H7), 3.98 (1H, d, H19, J = 12 Hz), 4.06 (1H, d, H19, J = 12 Hz), 4.37 (1H, brs, H3α, W½ = 7 Hz), 6.91 (1H, brs, H14), 7.02 (1H, brd, H12), 7.19 (1H, d, H11); MS m/z (rel. intensity) 432 (0.2), 430 (0.9), 428 (0.3), 417 (0.2), 415 (0.6), 413 (0.1), 399 (2.3), 397 (4.2), 395 (1.8), 350 (0.9), 348 (0.3), 335 (1.7), 333 (1.9), 317 (4.0), 315 (2.2), 270 (23.2), 255 (11.6), 252 (20.1), 237 (100.0), 211 (36.3), 195 (86.5), 178 (50.6), 165 (91.7), 153 (66.0), 141 (76.2), 128 (78.3), 115 (89.7); HRMS calcd. for C₁₉H₂₆OBr₂: 428.0387; found: 428.0313.

18-Norabiet-2, 4(19), 8, 11, 13-pentaene (197):

IR (neat) cm⁻¹ 2960 (CH), 1650, 1610 (C=C); ¹H NMR (CDCl₃) δ 1.01 (3H, s, C10 CH₃), 1.23 (6H, d, CH(CH₃)₂), 1.60 - 2.92 (m, aliphatic H), 4.95 (2H, d, H19), 5.80 (1H, m, H2), 6.23 (1H, brd, H3), 6.92 (1H, brs, H14), 7.04 (1H, brd, H12), 7.21 (1H, d, H11); MS m/z (rel. intensity) 252 (27.2), 237 (52.4), 209 (14.3), 195 (100.0), 178 (18.8), 171 (13.5), 167 (25.0), 165 (23.5), 152 (11.3), 141 (12.3), 129 (14.1), 115 (13.3).

18-Norabiet-4(19), 8, 11, 13-tetraen-3α-yl formate (198):

Trifluoromethanesulphonic anhydride (35.0 µL, 0.21 mmol) was added gradually to a stirred solution of pyridine (17.0 µL, 0.21 mmol) in methylene chloride (1 mL) at -23°C under argon. A solution of the allylic alcohol, 82 (48.50 mg, 0.18 mmol) in methylene chloride (1 mL) was added and the reaction mixture was stirred for 1 hour at -23°C. A further 35 µL of the anhydride were added and the reaction mixture was stirred for a further 0.5 hours. Potassium cyanide (35.10 mg, 0.54 mmol) and dimethylformamide (1 mL) were added at 23°C and the reaction mixture was stirred at room temperature for 17
hours. Water (10 mL) was added and the reaction mixture was extracted with ether (3×10 mL). The organic layers were combined, washed with water (3×5 mL) and brine (5 mL) and dried over sodium sulphate (anhydrous). The solvent was evaporated and the residue was separated by preparative TLC (Merck silica F_{254}, 0.5 mm) using hexanes-ethyl acetate 9:1 to give 12.90 mg (28.4%) of the olefin, 197 and 13.00 mg (24.4%) of the formate ester, 198, as a colourless oil. IR (neat) cm⁻¹ 2975 (CH), 1735 (C=O), 1665 (C=C), 1190 (C-O), ¹H NMR (CDCl₃) δ 1.01 (3H, s, C₁₀ CH₃), 1.23 (6H, d, CH(CH₃)₂), 1.68 - 2.17 (m, aliphatic H), 2.83 (1H, septet H₁₅), 2.92 (2H, m, H₇), 4.88 (1H, brs, H₁₉), 5.23 (1H, brs, H₁₉), 5.53 (1H, brs, OCHO), 6.94 (1H, brs, 1H, H₁₄), 7.02 (1H, brd, H₁₂), 7.21 (1H, d, H₁₁); MS m/z (rel. intensity) 298 (16.7), 283 (7.1), 252 (11.7), 237 (100.0), 224 (7.1), 209 (32.2), 195 (66.4), 178 (11.6), 167 (23.8), 153 (14.2), 141 (12.1), 129 (11.2), 115 (11.2); HRMS calcd. for C₂₀H₂₆O₂: 298.1933; found: 298.1939.

₄α, ₁₉α-Epoxy-1₈-norabieta-₈, ₁₁, ₁₃-trien-₃α-ol (199):

m-Chloroperbenzoic acid (1.80 g, 10.35 mmol) was added to a stirred solution of the allylic alcohol, 82, (1.40 g, 5.18 mmol) in methylene chloride (25 mL) at room temperature and the reaction mixture was stirred at room temperature for 5 hours. 10% sodium sulphite was added until the starch-iodide paper test was negative. The organic layer was washed with 5% sodium bicarbonate (2×5 mL), water (2×5 mL) and brine (5 mL) and dried over sodium sulphate (anhydrous). The solvent was evaporated to give 1.33 g (89.7%) of the epoxy alcohol, 199, as a white solid. mp 93-94°C; IR (CHCl₃) cm⁻¹ 3600, 3450 (OH), 3000 (CH₂O), 2950 (CH); ¹H NMR (CDCl₃) δ 1.15 (3H, s, C₁₀ CH₃), 1.23 (6H, d, CH(CH₃)₂), 1.32 - 2.52 (m, aliphatic H), 1.94 (1H, brs, OH), 2.73 (1H, d, H₁₉, J = 3 Hz), 2.85 (3H, m, H₇, H₁₅), 2.92 (1H, d, H₁₉, J = 3 Hz), 3.48 (1H, brs, H₃β), 6.92 (1H, brs, H₁₄), 7.02 (1H, brd, H₁₂), 7.21 (1H, d, H₁₁); MS m/z (rel. intensity) 286
19-Chloro-18-norabieta-8,11,13-trien-3α,4α-diol (200):

*p*-Toluenesulphonyl chloride (29.80 mg, 0.16 mmol) was added to a stirred solution of the epoxy alcohol, 199 (22.40 mg, 0.08 mmol) in pyridine (1 mL) at 0°C and the reaction mixture was stirred at 0°C overnight, followed by stirring at room temperature overnight. TLC analysis indicated that no reaction had taken place. The reaction mixture was stirred overnight at 105°C, poured into ice-water and and extracted with ether (40 mL). The organic layer was washed with 6N HCl (10 mL), 5% sodium bicarbonate (2x10 mL), water (10 mL) and brine (10 mL) and dried over sodium sulphate (anhydrous). The solvent was evaporated and the residue was purified by preparative TLC (Merck silica F_{254}, 0.5 mm) using hexanes-ethyl acetate 4:1 to give 15.9 mg (63.4%) of 200 as a white solid. IR (CHCl₃) cm⁻¹ 3640 (OH), 3550(OH), 2960 (CH); ¹H NMR (CDCl₃) δ 1.16 (3H, s, C₁₀ CH₃), 1.22 (6H, d, CH(CH₃)₂), 1.56 - 2.30 (m, aliphatic H), 2.39 (1H, brs, OH), 2.66 (1H, s, OH), 2.83 (1H, septet, H₁₅), 2.93 (2H, m, H₇), 3.78 (1H, d, H₁₉, J = 12 Hz), 3.93 (1H, d, H₁₉, J = 12 Hz), 4.10 (1H, brs, H₃β), 6.90 (1H, brs, H₁₄), 7.01 (1H, brd, H₁₂), 7.18 (1H, d, H₁₁); MS m/z (rel. intensity) 324 (11.3), 322 (31.8), 309 (2.5), 307 (7.0), 291 (33.0), 289 (100.0), 271 (17.9), 265 (6.4), 263 (15.5), 253 (25.1), 240 (9.9), 237 (10.4), 229 (25.8), 211 (22.1), 193 (50.0), 187 (14.8), 169 (16.1), 157 (28.3), 143 (27.0), 129 (26.6), 117 (23.0); HRMS calcd. for C₁₉H₂₇ClO₂: 322.1739; found: 322.1708.

19-Norabieta-2,8,11,13-tetraen-19-al (201):

Trifluoromethanesulphonic anhydride (118.0 µL, 0.70 mmol) was added to a stirred solution of the epoxy alcohol, 199 (100.00 mg, 0.35 mmol) and pyridine in methylene
chloride (3 mL) at -40°C under argon and the reaction mixture was stirred at -40°C for 2
hours. TLC analysis showed complete disappearance of the starting material and the
appearance of 2 products. Ether (30 mL) was added and the organic layer was washed
with water (3×10 mL) and brine (5 mL) and dried over magnesium sulphate (anhydrous).
The solvent was evaporated and the residue was separated by preparative TLC (Merck
silica F<sub>254</sub>, 0.5 mm) using hexanes-ethyl acetate 4:1 to give 33.50 mg (33.5%) of the
starting material, 199 and 27.00 mg (28.8%) of the unsaturated aldehyde, 201, as a
colourless oil. IR (neat) cm<sup>-1</sup> 2950 (CH), 1720 (C=O); <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.15 (3H, s,
C10 CH<sub>3</sub>), 11.24 (6H, d, CH(CH<sub>3</sub>)<sub>2</sub>), 1.61 - 2.89 (m, aliphatic H), 2.05 (1H, dt, H4β, J = 3,
11 Hz), 5.63 (1H, brd, H3, J = 11 Hz), 6.02 (1H, m, H2), 6.92 (1H, brs, H14), 7.04 (1H,
brd, H12), 7.20 (1H, d, H11), 9.58 (1H, d, H19, J = 3 Hz); MS m/z (rel. intensity) 268
(38.7), 253 (54.8), 239 (21.4), 235 (13.0), 225 (13.5), 223 (25.2), 211 (13.1), 197 (12.6),
195 (13.5), 193 (26.0), 186 (57.6), 181 (100.0), 179 (41.2), 171 (27.4), 165 (34.4), 152
(22.1), 143 (30.3), 141 (34.1), 129 (41.8), 115 (37.9); HRMS calcd. for C<sub>19</sub>H<sub>24</sub>O: 268.1827; found: 268.1828.

18-Norabieta-4(19').8.11.13-tetraen-3-one (202):

Dimethylsulphoxide (2.4 mL, 34.02 mmol) was added to a stirred solution of oxalyl
chloride (1.48 mL, 17.01 mmol) in methylene chloride (15 mL) at -78°C under argon and
the reaction mixture was stirred for 3 minutes. A solution of the allylic alcohol, 82 (2.30 g,
8.51 mmol) in methylene chloride (10 mL) was added and the reaction mixture was stirred
at -78°C for a further 15 minutes. Triethylamine (9.48 mL) was added and the reaction
mixture was stirred at room temperature for 1 hour. Ether (100 mL) was added and the
organic layer was washed with 10% HCl (3×10 mL), 5% sodium bicarbonate (10 mL),
water (10 mL) and brine (10 mL) and dried over sodium sulphate (anhydrous). The
solvent was evaporated and the residue was purified by VLC (Merck silica 60G, 60 g).
Stepwise elution using i) hexanes; ii) hexanes-ethyl acetate 19:1 and ii) hexanes-ethyl
acetate 9:1 gave 1.04 g (45.6%) of the unsaturated ketone, 202, as an unstable colourless oil. IR (neat) cm\(^{-1}\) 2960 (CH), 1700 (C=O), 1625 (C=C); \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 1.15 (3H, s, C10 CH\(_3\)), 1.24 (6H, d, CH(CH\(_3\))\(_2\)), 1.69 - 2.70 (m, aliphatic H), 2.87 (1H, septet, H15), 2.94 (2H, m, H7), 5.22 (1H, brs, H19), 5.98 (1H, brs, H19), 6.98 (1H, brs, H14), 7.06 (1H, brd, H12), 7.28 (1H, d, H11); MS m/z (rel. intensity) 268 (27.8), 253 (35.3), 225 (7.8), 211 (100.0), 193 (9.4), 183 (11.9), 171 (5.1), 167 (29.8), 155 (21.0), 153 (21.7), 141 (29.4), 128 (25.4), 115 (26.4).

3-Diethylphosphonato-18(4\(^3\))abeo-abieta-8, 11, 13-tetraen-18-nitrile (203):

0.5 M Lithium cyanide in DMF (2.01 mL, 1.01 mmol) and diethyl cyanophosphonate (117.0 \(\mu\)L, 1.01 mmol) were added to a stirred solution of the ketone, 202 (90.00 mg, 0.34 mmol) in DMF at room temperature under argon and the golden yellow reaction mixture was stirred for 1.5 hours. Water (10 mL) was added and the reaction mixture was extracted with ether (3\(\times\)10 mL). The organic layer was washed with water (3\(\times\)5 mL) and brine (5 mL) and dried over magnesium sulphate (anhydrous). The solvent was evaporated and the yellow-brown residue was purified by preparative TLC (Merck silica F\(_{254}\), 2 mm) using toluene-ethyl acetate 9:1 to give 85.40 mg (59.0%) of 203 as a yellow oil. IR (neat) cm\(^{-1}\) 2961 (CH), 1654 (C=C), 1278 (P=O), 1034 (P-O); \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 1.02 (3H, s, C10 CH\(_3\)), 1.24 (6H, d, CH(CH\(_3\))\(_2\)), 1.40 (6H, m, CH\(_3\)CH\(_2\)O), 1.83 - 3.01 (m, aliphatic H), 4.25 (4H, m, CH\(_3\)CH\(_2\)O), 5.05 (1H, brs, H19), 6.97 (1H, brs, H14), 7.04 (1H, brd, H12), 7.18 (1H, d, H11); DCI MS m/z (rel. intensity) 432 (3.23, M\(^+\) +1), 405 (5.91), 277 (1.61), 234 (5.91), 220 (2.69), 155 (100.0).

4\(\alpha\), 19\(\alpha\)-Epoxy-18-norabieta-8, 11, 13-trien-3-one (204):

Pyridinium chlorochromate (2.63 g, 12.22 mmol) was added to a stirred solution of the epoxy alcohol, 199 (700.00 mg, 2.44 mmol) in methylene chloride (50 mL) and the
reaction mixture was stirred at room temperature for 17 hours. The solvent was evaporated and the residue was suspended in ether and filtered through silica gel (Merck silica 230-400 Å, 10 mL) in a sintered glass funnel and the silica gel was washed with ether. The filtrate and the washings were combined and the solvent was evaporated to give 625.60 mg (90.0%) of the epoxy ketone, 204, as a colourless oil which solidified on standing. mp 76-78°C; IR (CHCl₃) cm⁻¹ 300 (CH-O), 2950 (CH), 1720 (C=O); ¹H NMR (CDCl₃) δ 1.24 (6H, d, CH(CH₃)₂), 1.37 (3H, s, C₁₀ CH₃), 1.42 - 2.88 (m, aliphatic H), 2.80 (1H, d, H₁₉, J = 5 Hz), 2.96 (1H, d, H₁₉, J = 5 Hz), 6.97 (1H, brs, H₁₄), 7.07 (1H, brd, H₁₂), 7.27 (1H, d, H₁₁); MS m/z (rel. intensity) 284 (66.5), 269 (100.0), 253 (11.7), 241 (26.1), 227 (24.3), 222 (19.6), 211 (36.1), 199 (49.4), 181 (31.3), 167 (47.3), 155 (32.1), 141 (41.4), 129 (45.3), 115 (36.4); HRMS calcd. for C₁₉H₂₄O₂: 284.1776; found: 284.1776; Anal. calcd. for C₁₉H₂₄O₂: C 80.24, H 8.51; found: C 80.04, H 8.41.

4α, 19α-Epoxy-3-cyano-18-norabeta-8, 11, 13-trien-3-ol (205):

2, 4, 6-Triisopropylbenzenesulphonyl hydrazide (63.00 mg, 0.21 mmol) was added to a stirred solution of the epoxy ketone, 204 (50.00 mg, 0.18 mmol) in THF (3 mL) under nitrogen at room temperature and the reaction mixture was stirred at room temperature for 2 hours. Potassium cyanide (34.40 mg, 0.53 mmol) and dicyclohexano-18-crown-6 (19.70 mg, 0.05 mmol) were added and the reaction mixture was stirred at 60°C for 1.5 hours. Ether (30 mL) was added and the organic layer was washed with water (3×10 mL), brine (5 mL) and dried over magnesium sulphate (anhydrous). The solvent was evaporated and the yellow oil was purified by column chromatography (Merck silica 230-400 Å, 10 g) using hexanes-ethyl acetate 4:1 to give 22.90 mg (41.9%) of the cyanohydrin, 205, as an unstable colourless oil. IR (neat) cm⁻¹ 3414 (OH), 2957 (CH); ¹H NMR (CDCl₃) δ 1.25 (6H, d, CH(CH₃)₂), 1.38 (3H, s, C₁₀ CH₃), 1.42 - 2.92 (m, aliphatic H), 2.81 (1H, d, H₁₉, J = 6 Hz), 2.96 (1H, d H₁₉ J = 6 Hz), 3.02 (1H, brs, OH),
6.97 (1H, brs, H14), 7.07 (1H, brd, H12), 7.27 (1H, d, H11); MS m/z (rel. intensity) 311 (0.3), 296 (1.5), 284 (56.1), 269 (83.2), 253 (11.7), 241 (15.6), 227 (16.3), 211 (27.5), 199 (30.2), 181 (17.9), 169 (20.4), 155 (15.3), 141 (18.1), 129 (19.1), 115 (12.9), 43 (100.0).

Ozonolysis of the exocyclic olefin, 81:

Ozone was passed into a stirred solution of the olefin, 81 (4.50 g, 17.69 mmol) in methanol-methylene chloride 5:1 (200 mL) at -78°C until the solution turned a pale blue colour (1 hour). The reaction mixture was stirred for a further 30 minutes at -78°C and dimethyl sulphide (1.6 mL, 21.23 mmol) was added. The reaction mixture was stirred at room temperature for 20 hours and the solvent was evaporated. The residue was dissolved in hexanes-ether 2:1 (150 mL) and washed with water (3×20 mL) and brine (10 mL). The aqueous layer was extracted with ether (50 mL) and the organic layers were combined and dried over magnesium sulphate (anhydrous). The solvent was evaporated and the yellow residue was separated by column chromatography (Merck silica 230-400 Å, 100 g) using hexanes-ethyl acetate 9:1 to give 3.00 g (66.2%) of the ketone, 206, as a white solid and 0.90 g (18.8%) of the diketone, 207, as a white solid.

18. 19-Dinorabieta-8, 11, 13-trien-4-one (206):

mp 40-42°C; IR (CHCl₃) cm⁻¹ 2950 (CH), 1710 (C=O); ¹H NMR (CDCl₃) δ 1.06 (3H, s, C₁₀ CH₃), 1.24 (6H, d, CH(CH₃)₂), 1.76 - 2.94 (m, aliphatic H), 6.95 (1H, brs, H14), 7.04 (1H, brd, H12), 7.22 (1H, d, H11); MS m/z (rel. intensity) 256 (33.3), 241 (100.0), 223 (16.6), 213 (21.3), 199 (10.1), 181 (22.9), 171 (13.2), 163 (5.2), 155 (5.8), 143 (10.0), 129 (15.6), 115 (5.4); HRMS calcd. for C₁₈H₂₄O: 256.1827; found: 256.1826; Anal. calcd. for C₁₈H₂₄O: C 84.33, H 9.43; found: C 84.30, H 9.21.
18, 19-Dinorabieta-8, 11, 13-trien-4, 7-dione (207):

mp 107-108°C; IR (CHCl₃) cm⁻¹ 2950 (CH), 1715 (C=O), 1680 (C=O); ¹H NMR δ 1.17 (3H, s, C₁₀ CH₃), 1.27 (6H, d, CH(CH₃)₂), 1.98 - 3.30 (m, aliphatic H), 7.38 (1H, d, H₁₁, J = 8 Hz), 7.45 (1H, dd, H₁₂, J = 3, 8 Hz), 7.95 (1H, d, H₁₄, J = 3 Hz); MS m/z (rel. intensity) 270 (35.6), 255 (100.0), 229 (9.5), 227 (11.8), 213 (30.5), 201 (15.7), 199 (14.0), 185 (24.9), 173 (16.0), 167 (6.5), 157 (10.5), 143 (14.6), 129 (14.1), 115 (10.8), 111 (16.1); HRMS calcd. for C₁₈H₂₂O₂: 270.1620; found: 270.1613; Anal. calcd. for C₁₈H₂₂O₂: C 79.96, H 8.20; found: C 80.18, H 8.23.

3-Dimethylthiomethylene-18, 19-dinorabieta-8, 11, 13-trien-4-one (208):

ₙ-Butyllithium (1.60 M, 15.2 mL, 24.27 mmol) was added to a stirred solution of 4-methyl-2, 6-di-ₙ-butylphenol (5.35 g, 24.27 mmol) in THF (100 mL) at 0°C under argon. Carbon disulphide (4.9 mL, 81.55 mmol) was added and the reaction mixture was allowed to warm to room temperature. A solution of the ketone, 206 (2.48 g, 9.70 mmol) in THF (25 mL) was added and the reaction mixture was stirred at room temperature for 48 hours. Methyl iodide (3.3 mL, 52.42 mmol) was added to the reaction mixture and stirring was continued at room temperature for a further 20 hours. The solvent was evaporated and the residue was dissolved in ether (200 mL). The ethereal solution was washed with water (3x20 mL) and brine (20 mL) and dried over magnesium sulphate (anhydrous). The solvent was evaporated and the orange residue was purified by column chromatography (Merck silica 230-400 Å, 100 g) using hexanes, followed by hexanes-ethyl acetate 9:1, to give 3.46 g (99.2%) of the ketene thioacetal, 208, as an orange oil. IR (neat) cm⁻¹ 2950 (CH), 1660 (C=O); ¹H NMR (CDCl₃) δ 1.1 (3H, s, C₁₀ CH₃), 1.23 (6H, d, CH(CH₃)₂), 1.76 - 3.42 (m, aliphatic H), 2.37 (3H, s, CH₃-S), 2.39 (3H, s, CH₃-S), 6.95 (1H, brs, H₁₄), 7.04 (1H, brd, H₁₂), 7.22 (1H, d, H₁₁); MS m/z (rel. intensity) 360 (15.7), 345
(27.1), 313 (10.3), 297 (5.8), 256 (24.7), 253 (71.1), 241 (62.4), 220 (20.8), 213 (19.0),
205 (81.8), 141 (22.5), 129 (29.3), 115 (22.1), 91 (100.0); HRMS calcd. for C_{21}H_{28}OS_{2}:
360.1582; found: 360.1587; Anal. calcd. for C_{21}H_{28}OS_{2}: C 69.95, H 7.83, S 17.78; found: C
69.97, H 8.00, S 17.60.

19- Hydroxy-18(4→3)abieta-3, 8, 11, 13-tetraen-18-oic acid lactone (91):

\[ n\text{-Butyllithium (1.60 M, 7.11 mL, 11.38 mmol) was added to a stirred suspension of trimethylsulphonium iodide (2.49 g, 12.19 mmol) in THF (50 mL) at -20^\circC under argon and the reaction mixture was allowed to warm to -10^\circC over 30 minutes. A solution of the ketene thioacetal, 208 (2.93 g, 0.81 mmol) in THF (15 mL) was added and the reaction mixture was stirred at -10^\circC for 10 minutes and at room temperature for 1.5 hours. The solvent was evaporated and the residue was dissolved in ether (200 mL). The ethereal solution was washed with water (2×20 mL) and the solvent was evaporated. The residue was dissolved in methanol (50 mL) and acetonitrile (25 mL) and concentrated HCl (6 mL) was added. The reaction mixture was stirred at room temperature for 40 hours and the methanol and acetonitrile were evaporated. The residual suspension was extracted into ether (200 mL and 2×50 mL) and the organic layer was washed with saturated sodium bicarbonate (3×30 mL) and dried over magnesium sulphate (anhydrous). The solvent was evaporated and the orangey-brown residue was purified by column chromatography (Merck silica 230-400 Å, 100 g) using hexanes-ethyl acetate 4:1 to give 1.02 g (42.3%) of the butenolide, 91, as a light yellow oil. IR (neat) cm\(^{-1}\) 2959 (CH), 1757 (C=O), 1678 (C=C); \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 1.02 (3H, s, C\(_{10}\) CH\(_3\)), 1.24 (6H, d, CH(CH\(_3\))\(_2\)), 1.71 - 2.73 (m, aliphatic H), 2.86 (1H, septet, H\(_{15}\)), 3.02 (2H, m, H\(_7\)), 4.78 (2H, m, H\(_{19}\)), 6.99 (1H, brs, H\(_{14}\)), 7.06 (1H, brd, H\(_{12}\)), 7.27 (1H, d, H\(_{11}\)); MS m/z (rel. intensity) 296 (31.1), 281 (100.0), 239 (16.9); HRMS calcd. for C\(_{20}\)H\(_{24}\)O\(_2\): 296.1777; found: 296.1772.\]

14C-Trimethylsulphonium iodide: 1.0 mCi of 14C-methyl iodide (in a break-seal vial) was cooled to -78°C and the seal was broken. Dimethyl sulphide (15.3 µL, 0.21 mmol) was added immediately and the vial was sealed with a rubber septum. Unlabelled methyl iodide (13.0 µL, 0.21 mmol) was added at room temperature and the trimethylsulphonium iodide was allowed to crystallise at room temperature overnight. The crystalline product was dried under vacuum for 2 hours immediately before use.

Synthesis of the [19-14C] butenolide: n-Butyllithium (1.60 M, 121.0 µL, 0.19 mmol) was added to a stirred suspension of 14C-trimethylsulphonium iodide (0.21 mmol) in THF (1 mL) at -16°C under argon. The reaction mixture was allowed to warm to -10°C over 12 minutes and a solution of the ketene thioacetal, 208 (49.40 mg, 0.14 mmol) in THF (1 mL) was added. The reaction mixture was stirred at -10°C for 10 minutes and at room temperature for 1.5 hours. The solvent was evaporated and the residue was partitioned between ether (25 mL) and water (3 mL). The organic layer was washed with water (2×3 mL) and the aqueous layers were combined and extracted with a further 10 mL of ether. The organic layers were combined and the solvent was evaporated. Methanol (1 mL) and acetonitrile (2 mL) were added to the residue and the solution was cooled to 0°C. Concentrated HCl (0.3 mL) was added and the reaction mixture was stirred at room temperature for 24 hours. The solvent was evaporated and the orange residue was purified by column chromatography (Merck silica 230-400 Å, 5 g) using hexanes-ethyl acetate 4:1 to give 6.18 mg of the radioactive butenolide, 209, as a light yellow oil. The radioactive butenolide, 209, was diluted to 297.68 mg with unlabelled butenolide, 91, and counted. Activity 1.22×10^7 DPM (0.6% incorporation); specific activity 5.47 mCi mol⁻¹ (1.21×10^{10} DPM mol⁻¹).
3.10 Biotransformation studies of the butenolide, 91, and the radioactive butenolide, 209

3.10.1 Time course studies using the radioactive butenolide, 209

Addition of the butenolide at the end of the growth phase:

TRP-4a suspension cultures were grown as described previously in MSNA0.5K0.5 production medium until the stationary phase was reached (24 days, RI 1.3335). Portions of the radioactive butenolide, 209, were added to 100 mL samples of the culture and the samples were incubated for a further specified amount of time: sample 1 (10.13 mg 4.14×10^5 DPM) was incubated for 24 hours; sample 2 (10.05 mg 4.11×10^5 DPM) was incubated for 48 hours and sample 3 (10.12 mg 4.14×10^5 DPM) was incubated for 72 hours. At the end of the incubation period, the samples were harvested and extracted. Control samples (no precursor was added) of 100 mL each were also harvested at 24, 48 and 72 hours and extracted.

A blank experiment was conducted in which the unlabelled butenolide, 91 (14.89 mg), was incubated with 250 mL of the MSNA0.5K0.5 medium (without cells) for 72 hours.

Extraction procedure:

The cells and the spent medium were homogenized together using an Ultra-Turrax T25 disperser (Janke and Kunkel) and filtered through celite. The filtrate was saturated with sodium chloride and extracted with ethyl acetate (2×100 mL). The cell debris was sonicated with ethyl acetate (50 mL) for 30 minutes, filtered through celite, and the celite was washed with ethyl acetate. The organic layers (from the spent medium and the cell debris) were combined, dried over sodium sulphate (anhydrous), and the solvent was evaporated. Sample 1 (24 hours) gave a crude brown extract (52.21 mg, 3.41×10^5 DPM);
sample 2 (48 hours) gave a crude brown extract (51.81 mg, 3.37×10^5 DPM); and sample 3 (72 hours) gave a crude brown extract (51.79 mg, 3.44×10^5 DPM). Control 1 (24 hours) gave a crude brown extract (39.29 mg); control 2 (48 hours) gave a crude brown extract (40.53 mg); and control 3 (72 hours) gave a crude brown extract (36.54 mg). The results are presented in Table 19.

The blank experiment was extracted with ethyl acetate (3×100 mL) to give the butenolide, 91 (14.18 mg, 95.2% recovery), unchanged.

TLC analyses:

The samples and the controls were analysed by TLC (toluene-ethyl acetate 4:1; chloroform-methanol-acetic acid 95:5:1) and showed the presence of tripdiolide (1) and triptolide (2). The samples showed also the presence of starting precursor and an additional metabolite which was strongly UV absorbing. The controls showed no strongly UV absorbing metabolites.

Radioactivity profiles:

Sample 1 (3.20 mg, 2.09×10^4 DPM) was applied to an analytical TLC plate (Merck silica F_{254}, 20×20 cm, 0.25 mm with concentrating zone) and eluted with toluene-ethyl acetate 4:1. A small strip of the plate was cut and visualized using anisaldehyde-H_2SO_4. Bands were cut according to the positions of the visualized spots and the silica gel from each band was scraped directly into scintillation vials. Methanol (0.5 mL), water (0.5 mL) and scintillation cocktail (10 mL) were added to each vial and the vials were sonicated for 10 minutes and counted. The results are presented in Table 20 and Figure 29.

The procedure was repeated for sample 2 (3.77 mg, 2.45×10^4 DPM) and sample 3 (3.84 mg, 2.55×10^4 DPM). The results are presented in Table 21, Figure 30 and Table 22, Figure 31, respectively.
Addition of the butenolide at day 11 after resuspension in MSNA$_{0.5}$K$_{0.5}$:

A solution of the radioactive butenolide, 209 (10.61 mg, 4.34x10$^5$ DPM) in ethanol (1 mL) was added to 200 mL of TRP-4a suspension culture grown in MSNA$_{0.5}$K$_{0.5}$ for 11 days and the culture was incubated until the end of the growth phase (15 days, 26 days after resuspension in MSNA$_{0.5}$K$_{0.5}$). The culture was suction filtered and the cells were extracted by homogenization in 10% methanol-ethyl acetate (200 mL) using an Ultra-Turrax T25 disperser. The cells were filtered and the cell debris was washed with ethyl acetate. The aqueous filtrate (spent medium) was saturated with sodium chloride and extracted with ethyl acetate (2x100 mL). The organic layers (from the spent medium and the cells) were combined and the solvent was evaporated to give a brown extract (176.90 mg, 3.43x10$^5$ DPM).

Radioactivity profiles:

Radioactivity profiles of the extract were obtained using the procedure outlined above. The profiles were run using 8.04 mg (1.56x10$^4$ DPM) of the extract (the TLC plate was eluted twice with methylene chloride-ethyl acetate 1:1) and 9.52 mg (1.85x10$^4$ DPM) of the extract (the TLC plate was eluted with toluene-ethyl acetate 4:1). The results are presented in Table 23, Figure 32 and Table 24, Figure 33, respectively.

3.10.2 Large scale biotransformation of the radioactive butenolide, 209, and the inactive butenolide, 91:

The TRP-4a suspension cultures were grown as previously, resuspended in MSNA$_{0.5}$K$_{0.5}$ production medium and incubated for 12 days. A solution of the radioactive butenolide, 209 (101.26 mg, 4.14x10$^6$ DPM) in ethanol (2 mL) was added to 2 L of the suspension culture (in 500 mL batches) and a solution of the inactive butenolide, 91
(301.28 mg) in ethanol (6 mL) was added to 6 L of the suspension culture.(in 500 mL batches) The suspension cultures were incubated until the stationary phase was reached (a further 20 days, 32 days after resuspension in MSNA$_{0.5}$K$_{0.5}$) and harvested (500 mL of the culture incubated with the radioactive precursor was found to be contaminated and was discarded).

**Extraction of the radioactive culture:**

The remaining 1.5 L of radioactive culture was suction filtered and the cells were homogenized in ethyl acetate (1 L) using an Ultra-Turrax T25 disperser. The cell debris was washed with ethyl acetate and discarded. The filtered spent medium was saturated with sodium chloride and extracted with ethyl acetate (2×1 L). The organic layers from the cells and the spent medium were combined and the solvent was evaporated to give an orangey-brown extract (781.83 mg, 2.93×10$^6$ DPM).

**Extraction of the inactive culture:**

The inactive culture was filtered and the spent medium was freeze-dried. The cells were homogenized in ethyl acetate (1.5 L) using an Ultra-Turrax T25 disperser, filtered and the cell debris was washed with ethyl acetate. The freeze-dried spent medium was reconstituted with water (500 mL) and extracted with ethyl acetate (2×500 mL). The organic layers from the cells and the spent medium were combined and the solvent was evaporated to give an orangey-brown extract (2.56 g).

**Isolation of the radioactive metabolites from the radioactive extract:**

The radioactive extract (781.83 mg, 2.93×10$^6$ DPM) was separated by column chromatography (Merck silica 230-400 Å, 80 g) using toluene-ethyl acetate 4:1 and rapid
elution with ethyl acetate and methanol to give 8 fractions. Fraction 1 (103.86 mg, 4.53×10^5 DPM) contained unchanged butenolide, 209, while fractions 2 and 3 (65.45 mg, 1.15×10^6 DPM and 8.52 mg, 9.54×10^4 DPM, respectively) contained the benzylic ketone, 210. Fractions 4 and 5 (83.59 mg, 4.99×10^5 DPM and 77.54 mg, 2.60×10^5 DPM, respectively) contained the hydroxy ketone, 211, the benzylic alcohol, 212 and the alcohol, 213. Fraction 6 (25.16 mg, 1.16×10^5 DPM) was eluted with ethyl acetate and contained a small quantity of the alcohol, 213 and tripdiolide, 1. Fraction 7 (16.60 mg 4.37×10^4 DPM) was eluted with ethyl acetate, while fraction 8 (375.24 mg, 2.26×10^4 DPM) was eluted with methanol.

**Isolation of the radioactive butenolide, 209:**

Fraction 1 (103.86 mg, 4.53×10^5 DPM) was separated by column chromatography (Merck silica 230-400 Å, 10 g) using hexanes-ethyl acetate 4:1 to give the radioactive butenolide, 209 (13.01 mg, 4.53×10^5 DPM), as a light yellow oil.

**Isolation of the radioactive benzylic ketone, 210:**

Fractions 2 and 3 were combined (73.97 mg, 1.25×10^6 DPM) and separated by column chromatography (Merck silica 230-400 Å, 8 g) using toluene-ethyl acetate 9:1 to give the benzylic ketone, 210 (36.42 mg, 1.31×10^6 DPM), as a light yellow foam.

**Isolation of 211, 212, 213 and tripdiolide (1):**

Fractions 4, 5 and 6 were combined (186.29 mg, 8.74×10^5 DPM) and separated by column chromatography (Merck silica 230-400 Å, 18 g) using methylene chloride-ethyl acetate 5:1 to give 5 fractions. Fractions 1, 2 and 3 (33.20 mg, 9.66×10^4 DPM, 22.35 mg, 2.65×10^5 DPM and 9.12 mg 7.20×10^4 DPM, respectively) were separated further by
preparative TLC (Merck silica F$_{254}$, 0.5 mm, 3 plates, toluene-ethyl acetate-chloroform-formic acid 35:15:16:1, the plates were eluted twice) to give 211 (5.19 mg, 1.37×10$^5$ DPM) and 212 (8.07 mg, 2.21×10$^5$ DPM), as yellow solids. Fractions 4 and 5 (17.67 mg, 4.21×10$^4$ DPM and 11.51 mg, 2.03×10$^5$ DPM, respectively) were combined and separated further by preparative TLC (Merck silica F$_{254}$, 0.5 mm, benzene-methanol-acetic acid 85:10:5, the plate was eluted twice) to give 213 (4.69 mg, 1.58×10$^5$ DPM) and tripdiolide (1, 2.72 mg, 2.97×10$^4$ DPM). The results are presented in Table 25.

Isolation of the inactive metabolites from the inactive extract:

The inactive extract (2.56 g) was separated by column chromatography (Merck silica 230-400 Å, 100 g) using toluene-ethyl acetate 4:1 and rapid elution with ethyl acetate to give 7 crude fractions. Fraction 1 (409.97 mg) contained the starting material, 91, while fraction 2 (140.09 mg) contained the benzylic ketone, 214. Fractions 3 and 4 (37.50 mg and 96.32 mg, respectively) contained only indigenous metabolites. Fractions 5 and 6 (116.09 mg and 468.02 mg, respectively) contained the hydroxy ketone, 215, the benzylic alcohol, 216 and the C2 alcohol, 96.

Isolation of the butenolide, 91:

Fraction 1 was separated by column chromatography (Merck silica 230-400 Å, 40 g) using hexanes-ethyl acetate 4:1 to give the starting material, 91 (64.13 mg), as a light yellow oil.

Isolation of the benzylic ketone, 214:

Fraction 2 was separated by preparative TLC (Merck silica F$_{254}$, 2 mm) using methylene chloride-ethyl acetate 5:1 (the plate was eluted twice) to give the benzylic ketone, 214 (104.36 mg), as a light yellow foam.
Isolation of 215, 216 and 96:

Fractions 5 and 6 were combined (584.51 mg) and separated by column chromatography (Merck silica 230-400 Å, 20 g) using methylene chloride-ethyl acetate 5:1 and rapid elution with ethyl acetate to give 10 fractions. Fraction 1 (127.37 mg) contained only indigenous metabolites while fractions 2 and 3 (39.26 mg and 41.96 mg, respectively) contained 215 and 216. Fractions 4 and 5 (30.98 mg and 39.66 mg, respectively) contained only indigenous metabolites and fractions 6 and 7 (54.68 mg and 14.59 mg, respectively) contained 96 and tripdiolide (1). Fractions 8, 9 and 10 (20.89 mg, 22.80 mg and 115.88 mg, respectively) contained only indigenous metabolites. Fractions 2 and 3 were separated further by preparative TLC (Merck silica F254, 0.5 mm, 2 plates) using toluene-ethyl acetate-chloroform-formic acid 35:15:16:1 (the plates were eluted twice) to give 215 (9.61 mg) as an off-white solid, and 216 (9.35 mg) as a white solid. Fractions 6 and 7 were separated further by preparative TLC (Merck silica F254, 0.5 mm, 2 plates, benzene-methanol-acetic acid 85:10:5, the plates were eluted twice) to give 96 (13.35 mg) as a white solid.

19-Hydroxy-7-oxo-18(4→3)abeo-abieta-3, 8, 11, 13-tetraen-18-oic acid lactone (214):

IR (CHCl3) cm⁻¹ 2950 (CH), 1745 (C=O), 1680 (C=O); ¹H NMR (CDCl₃) δ 1.17 (3H, s, C10 CH₃), 1.27 (6H, d, CH(CH₃)₂), 1.80 - 3.80 (m, aliphatic H), 2.99 (1H, septet, H15), 4.78 (2H, m, H19), 7.42 (1H, d, H11, J = 8 Hz), 7.58 (1H, dd, H12, J = 2, 8 Hz), 7.97 (1H, d, H14, J = 2 Hz); MS m/z (rel. intensity) 310 (60.3), 295 (100.0), 253 (7.2), 251 (3.6), 237 (3.0), 225 (5.2), 213 (38.6), 209 (11.4), 195 (3.0), 187 (42.7), 181 (8.5), 178 (10.1), 165 (22.4), 145 (16.2), 128 (26.4), 115 (32.3); HRMS calcd, for C₂₀H₂₂O₃: 310.1569; found: 310.1571.
2β, 19-Dihydroxy-7-oxo-18(4→3)_abeo_-abieta-3, 8, 11, 13-tetraen-18-oic acid lactone (215):

IR (CHCl₃) cm⁻¹ 3300 (OH, br), 1765 (C=O), 1690 (C=O); ¹H NMR (CDCl₃) δ 1.16 (3H, s, C10 CH₃), 1.28 (6H, d, CH(CH₃)₂), 1.60 (1H, brs, OH), 1.79 - 3.33 (m, aliphatic H), 2.98 (1H, septet, H15), 4.92 (1H, brs, H2α), 6.18 (2H, brd, H19), 7.40 (1H, d, H11, J = 8 Hz), 7.48 (1H, dd, H12, J = 2, 8 Hz), 7.97 (1H, d, H14, J = 2 Hz); MS m/z (rel. intensity) 326 (88.9), 324 (16.9), 311 (93.6), 309 (42.5), 298 (17.0), 295 (21.7), 293 (69.5), 283 (17.3), 280 (17.2), 265 (34.9), 251 (43.2), 237 (41.3), 223 (22.7), 213 (77.4), 200 (29.7), 195 (28.6), 187 (100.0), 165 (36.2), 145 (32.9), 141 (26.2), 128 (32.6), 115 (35.9); HRMS calcd. for C₂₀H₂₂O₄: 326.1518; found: 326.1514.

7β, 19-Dihydroxy-18(4→3)_abeo_-abieta-3, 8, 11, 13-tetraen-18-oic acid lactone (216):

mp 85-86°C; IR (CHCl₃) cm⁻¹ 3600 (OH), 2950 (CH), 1755 (C=O); ¹H NMR (CDCl₃) δ 1.13 (3H, s, C10 CH₃), 1.26 (6H, d, CH(CH₃)₂), 1.58 (1H, brs, OH), 1.66 - 2.85 (m, aliphatic H), 2.93 (1H, septet, H15), 4.80 (2H, brd, H19), 5.02 (1H, brt, H7α), 7.18 (1H, dd, H12, J = 2, 8 Hz), 7.29 (1H, d, H11, J = 8 Hz), 7.44 (1H, d, H14, J = 2 Hz); MS m/z (rel. intensity) 312 (63.2), 310 (15.3), 297 (31.1), 295 (22.2), 281 (14.8), 279 (36.9), 269 (100.0), 251 (10.1), 237 (95.7), 235 (22.9), 223 (7.5), 213 (11.6), 209 (13.5), 193 (36.5), 178 (30.3), 165 (28.9), 151 (37.6), 141 (25.9), 128 (25.9), 115 (28.4); HRMS calcd. for C₂₀H₂₄O₃: 312.1725; found: 312.1722.

2β, 19-Dihydroxy-18(4→3)_abeo_-abieta-3, 8, 11, 13-tetraen-18-oic acid lactone (96):

mp 187-188°C (dec); IR (CHCl₃) cm⁻¹ 3610 (OH), 2960 (CH), 1760 (C=O); ¹H NMR (CDCl₃) δ 1.26 (3H, s, C10 CH₃), 1.26 (6H, d, CH(CH₃)₂), 2.03 - 2.62 (m, aliphatic H), 2.11 (1H, brs, OH), 2.95 (1H, septet, H15), 4.84 (1H, m, H19), 5.03 (1H, m, H19), 5.17 (1H, t, H2α, J = 8 Hz), 7.21 (1H, dd, H12, J = 2, 8 Hz), 7.28 (1H, d, J = 8 Hz), 7.39 (1H, d, H14 J = 2 Hz); MS m/z (rel. intensity) 312 (3.5), 310 (95.8), 295 (34.8), 279
Reduction of 214:

Excess sodium borohydride was added to a stirred solution of 214 (9.30 mg, 0.03 mmol) in ethanol (2 mL) at room temperature and the reaction mixture was stirred at room temperature for 30 minutes. The solvent was evaporated and the residue was dissolved in ether (15 mL). The ethereal solution was washed with 10% HCl (3×5 mL) and water (2×5 mL) and the solvent was evaporated. The residue was purified by preparative TLC (Merck silica F254, 0.5 mm) using methylene chloride-ethyl acetate 5:1 to give 8.82 mg (94.2%) of a white solid which had identical TLC, mp, IR, MS and 1H NMR spectra to 216.

2, 4-Dinitrophenylhydrazone derivative of 210:

Concentrated H2SO4 (5 drops) was added to a stirred suspension of 2, 4-dinitrophenylhydrazine (63.30 mg, 0.13 mmol) in methanol (1 mL). A solution of 210 (36.42 mg, 0.12 mmol, 1.31×10^6 DPM) was added and the reaction mixture was warmed gently for 5 minutes. The resulting precipitate was suction filtered, washed with 10% HCl, water and methanol and dried to give 36.71 mg (63.8%) (8.27×10^5 DPM) of the hydrazone, 217, as an orange solid.

Recrystallization to constant activity of the 2, 4-dinitrophenylhydrazone, 217:

The 2, 4-Dinitrophenylhydrazone, 217 (36.71 mg, 8.27×10^5 DPM) was recrystallized from ethyl acetate and collected by suction filtration. The crystals were
dried and weighed and a sample was counted. Recrystallization was repeated until a constant specific activity was reached. The results are presented in Table 27.

Recrystallization to constant activity of the radioactive alcohol, 212:

The above procedure was followed for 212 (8.07 mg, 2.21×10^5 DPM), except that ether-hexanes was used as the recrystallising solvent system. The inactive alcohol, 216 (5.41 mg) was added after the first recrystallization (2.18 mg, 6.81×10^4 DPM, 3.13×10^4 DPM mg^{-1}) to give 7.59 mg (6.81×10^4 DPM, 8.98×10^3 DPM mg^{-1}). The results are presented in Table 27.

Recrystallization to constant activity of the radioactive alcohol, 213:

The above procedure was followed for 213 (4.69 mg, 1.58×10^5 DPM), except that hexanes-ethyl acetate was used as the recrystallising solvent system. The inactive alcohol, 96 (6.15 mg) was added before recrystallization to give 10.84 mg (1.58×10^5 DPM, 1.46×10^4 DPM mg^{-1}). The results are presented in Table 27.

Recrystallization of tripdiolide (1) isolated from the radioactive extract:

The tripdiolide fraction (2.72 mg, 2.97×10^4 DPM, 1.09×10^4 DPM mg^{-1}) was diluted with 5.62 mg of an authentic sample of tripdiolide (1) to give 8.34 mg (2.97×10^4 DPM, 3.56×10^3 DPM mg^{-1}) and recrystallized from ethanol. Recrystallization to a constant specific activity was carried out according to the above procedure and the results are presented in Table 27.
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